ON THE ANTIGENS OF RED BLOOD CORPUSCLES.

II. Flocculation Reactions with Alcoholic Extracts of Erythrocytes.*

By K. LANDSTEINER, M.D., and JAMES van der SCHEER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 14, 1925.)

In a previous paper[1] it was pointed out that not only the heterogenetic substance present in sheep blood (Sordelli and Pico, Sachs and Guth), but alcoholic extracts of erythrocytes in general give flocculation reactions with hemolytic immune sera.

A further study of these reactions was undertaken in the following experiments. It was stated previously that the strongest reactions were obtained with immune sera resulting from injections of alcoholic extracts of blood (mixed with serum), but that they also occurred with common anti-eryhrocyte sera. The first object was to obtain further information regarding the specificity of these reactions.

EXPERIMENTAL.

A rather large number of different hemolytic immune sera, which were at our disposal, were tested with emulsions of alcoholic extracts of various bloods (Table I).

The extracts and emulsions were prepared as follows: 20 cc. of oxalated blood was centrifuged, washed 3 times with saline solution, and to the sediment 50 cc. of 95 per cent alcohol was slowly added, while shaking, to prevent the formation of clumps. After standing for 24 hours at room temperature the alcohol solution was filtered, and the residue was again extracted in the same way with 20 cc. of 95 per cent alcohol. The combined alcoholic extracts were filtered and concentrated to 10 cc., corresponding to one-half the original blood volume. After cooling to room temperature this solution was filtered again to remove any turbidity. The term "alcoholic extract" used in this communication refers to this kind of solution, unless special mention is made of a change in technique.

---

* Twenty-first paper on antigens and specificity.

[1] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427; *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 170.

The emulsions used for the flocculation reactions were prepared in different ways. The original method employed by us, of adding 5 parts of saline, drop by drop, to 1 part of alcoholic extract (method of Sachs and Guth for heterogenetic antigen) could not be used equally well for all kinds of blood. In some cases the emulsions were not stable enough, and for this reason yielded too many weak heterologous reactions, undesirable for the study of the specificity. It was found possible to increase the stability of the emulsions by a quicker addition of the saline solution, and the most stable emulsions were those obtained by quickly adding the alcoholic extract to the salt solution (*cf.* Sachs). For each kind of blood the most suitable method of emulsification had to be determined by preliminary experiments. It even happened occasionally that extracts of bloods of the same species, and apparently made in the same way, showed differences in the stability of their emulsions. In the case of rat blood it was found necessary to use the acetone-insoluble fraction of the extract and to add cholesterol. After evaporating the alcoholic extracts to dryness, and redissolving the residue with boiling absolute alcohol, extracts were obtained which still reacted typically. Furthermore, filtration of the solution in absolute alcohol through Berkefeld filters did not remove the active substances. For each extract the tests with all the sera were made on the same day and with the same freshly prepared emulsion.

Preparation of the Emulsions Used in the Experiment of Table I.

1. Horse Blood.—To 1 part of alcoholic extract of blood 5 parts of saline were added, drop by drop while shaking.

2. Chicken Blood.—Prepared in the same manner, with addition of saline very slowly, drop by drop.

3. Rat Blood.—10 cc. of alcoholic extract, corresponding to 20 cc. of blood, was evaporated to dryness and extracted twice with 20 cc. of boiling acetone. The residue was extracted with 70 cc. of 95 per cent alcohol on the steam bath, the alcoholic solution filterd hot, concentrated to 10 cc., and again filtered after cooling. To 5 cc. of this alcoholic solution was added 0.3 cc. of a 1 per cent alcoholic cholesterol solution, in alcohol, and then 5 times the volume of saline, drop by drop. (Without cholesterol only weak reactions were obtained. The original alcoholic extract gave emulsions flocculating by themselves.)

4. Human Blood.—1 part of alcoholic extract was blown from a pipette into 5 parts of saline.

5. Rhesus Blood.—In the same way as 4.

6. Pig Blood.— " " " " " "

7. Sheep Blood.— " " " " " "

8. Horse Kidney.—1 part of minced horse kidney was extracted with 5 times the volume of 95 per cent alcohol for 48 hours, at room temperature. The alcoholic solution was filtered, and 1 part was blown into 5 parts of saline.

9. Guinea Pig Blood.—No entirely satisfactory method has been found for this blood extract. The alcoholic extract was diluted 5 times with 95 per cent

alcohol, and 1 part was blown into 5 parts of saline. In practically every tube, including the control, slight flocculations occurred. The table gives only the readings of the distinct reactions.

To 0.2 cc. of the inactivated rabbit immune sera, diluted one-half, 0.2 cc. of a given emulsion was added, and the tubes were kept at 37°C. for 20 hours.

In the readings both the formation of flakes and of sediment were taken into account. The strength of the reactions is indicated as follows: − = negative; F.tr. = faint trace; Tr. = trace; ±, +, +±, etc.; ? = questionable; blank spaces = no test made. Several times we noticed that the reactions had increased in intensity when the tubes were allowed to stand longer than the usual period of 20 hours. An example is given in the last column of the table (pig blood).

One sees from the experiments that a considerable number of the immune sera react distinctly on the emulsions of homologous blood extract, but to different degrees. In the case of the immune sera against human blood of Group I and horse blood, the reactions were comparatively weak. The differences in the effect of the individual immune sera are certainly due in part to their unequal hemolytic or agglutinative strength; but the existence of qualitative differences has also to be considered.[1]

Besides the homologous reactions others occurred. If one disregards the faint ones, these heterologous reactions display a certain regularity. An example is to be found in the frequency of reactions of horse, donkey, and mule blood antisera with emulsions of alcoholic extract of rat blood. Six out of ten immune sera for horse blood, five for donkey blood, and three out of ten for mule blood gave flocculations with rat blood extract, most of them of considerable strength, while none of the other heterologous sera reacted. That these reactions were mostly stronger than those on horse blood extract may be explained by the fact that cholesterol was used in the preparation of the rat blood extract emulsion, and not for the horse blood extract. There was no visible effect of the rat blood immune sera on horse blood extract. A similar case is to be found in the action of rhesus and baboon blood immune sera on pig blood extract. Moreover, it will be noted that the two immune sera against monkey blood also flocculate human blood extracts, while the reverse reaction is rare and faint. Another group of such reactions is that of the blood corpuscles known to be related to the so called "Forssman antigen;" *viz.*,

TABLE I.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Human blood Group I.	17	Tr.	F.tr.	−		−	−	−	−			
	18	−	−	−	−	−	−	−	−	−	−	−
	19	−	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	Tr.	−	−	−	−	−	+±
	24	−	−	−	−	−	−	−	−	−	−	−
	25	+±	Tr.	−	−	−	−	−	−	Tr.?	F.tr.	+
	26	−	−	−	−	−	−	−	−	−	−	−
	27	Tr.	Tr.?	−	−	−	−	−	−	−	−	−
	64	−	−	F.tr.	±	−	−	−	−	−	−	±
	65	−	−	−	−	−	−	−	−	−	−	Tr.
	4–63	−	F.tr.	−	−	−	−	−	−	−	−	−
	4–64	±	Tr.	−	−	−	−	−	−	F.tr.	−	−
Human blood Group II.	20	−	+±	+	−	−	−	−	−	−	−	−
	21	+	+++	+±	−	−	−	−	−	Tr.	−	+±
	22	±	+++	++±	−	−	−	−	−	−	−	+
	3–43	−	±	−	−	−	−	−	−	−	−	−
	3–44	−	F.tr.	Tr.	Tr.	−	−	−	−	−	−	−
	3–45	−	−	−	−	−	−	−	−	−	−	−
	3–46	−	F.tr.	F.tr.	−	Tr.	−	−	−	−	−	−
	4–14	−	++±	++	−	−	−	−	−	−	−	−
	4–15	−	F.tr.	F.tr.	−	−	−	−	−	−	−	−
	4–16	−	−	−	F.tr.	−	−	−	−	−	−	−
	4–17	−	−	F.tr.	−	−	−	−	−	−	−	−
	4–18	+	Tr.	−	−	−	−	−	−	−	−	F.tr.
	4–19	±	+	Tr.	−	±	−	−	−	−	−	+±

Rhesus blood.	4-56	++	+±	−	−	−	−	−	−	+±	Tr.	++
	4-67			−	−					++±	+±	++±
Baboon blood.	4-58	+	+±	Tr.	−	−	−	−	−	+±	+	++±
	4-59			−	−						Tr.	++
Sheep blood.	10	−	−	+±	+	−	−	−	Tr.			
	11	F.tr.	+++	+++	+++	++	−	−	F.tr.			
	12	F.tr.	−	++	++	Tr.	−	−	Tr.	Tr.?	−	F.tr.
Horse kidney.	54	−	+	+++	++±	++	F.tr.	−	−	−	−	Tr.
	4-02	−	−	+++	++±	++	F.tr.	−	+	Tr.	−	+
	4-03	−	−	+++	+	++	−	−	−	−	−	−
	4-04	−	−	+++	±	++	−	−	−	−	−	−
Beef blood.	4-32	−	−	−	−	−	−	−	−	−	−	−
Horse.	1	Tr.	−	−	−	−	±	+++	−			
	3	−	−	−	−	−	±	−	−	−	−	−
	30	−	−	−	−	−	Tr.	++	−	−	−	−
	31	−	−	−	−	−	Tr.?	−	−	−	−	−
	32	−	−	F.tr.	F.tr.	−	+	±	−	Tr.?	−	F.tr.
	33	−	F.tr.	−	−	−	−	++	−	−	−	+
	34	−	−	−	−	−	+	+++	−	−	−	−
	35	−	−	F.tr.	−	−	Tr.	+++	−	−	−	−
	2-62	−	−	−	−	−	Tr.	−	−	−	−	−
	3-03	−	−	−	−	−	−	−	−	−	−	−
Donkey blood.	36	−	−	−	−	−	±	+++	−	−	−	−
	37	−	−	−	−	−	++	+++	±	−	−	−
	38	−	−	−	−	−	−	+	−	−	−	−
	44	−	−	−	−	−	−	++	−	−	−	−
	2-74	−	−	−	−	−	Tr.?	F.tr.	−	−	−	−

*Same tests as in the preceding column after standing 1 more day at room temperature.

TABLE I—*Concluded.*

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Mule blood.	60	–	–	–	–	–	–	–	–	–	–	–
	61	–	–	–	–	–	–	–	–	–	–	–
	74	–	–	–	–	–	–	–	–	–	–	–
	76	–	–	–	–	–	–	–	–	–	–	–
	3–05	–	–	–	–	–	–	–	–	F.tr.	–	–
	3–07	–	–	–	–	–	–	–	–	–	–	–
	3–08	–	–	–	–	–	F.tr.	–	–	–	–	–
	3–32	–	–	F.tr.	Tr.	–	Tr.	+±	–	–	–	–
	3–34	–	–	–	–	–	++	++	–	–	–	Tr.
	3–39	–	–	–	–	–	++	++	–	–	–	–
Rat blood.	29	–	–	F.tr.	–	–	–	+++	+	–	–	F.tr.
	66	–	–	–	–	–	–	–	–	–	–	–
	67	–	F.tr.	F.tr.	Tr.	–	–	+++	–	–	–	–
	77	–	–	–	–	–	–	–	–	–	–	–
	79	–	–	–	–	–	–	–	–	Tr.?	–	–
	80	–	–	–	–	–	–	–	–	–	–	Tr.
	86							++				
	88	–	–	–	–	–	–	±	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	–
Guinea pig blood.	8	–	–	–	–	–	–	–	++	–	–	–
	21	–	F.tr.	–	–	–	–	–	++	–	–	–
	68	–	–	–	–	–	–	–	++	–	–	–

Chicken blood.	41	−	−	+++	±	++±	F.tr.	−	−	−	−	−
	43	−	−	+++	F.tr.	±	−	−	−	−	−	−
	3–64	−	−	+++	±	+±	−	−	−	−	−	−
Duck blood.	3–58	−	Tr.	Tr.	F.tr.	Tr.	Tr.	−	Tr.	−	−	Tr.
	3–59	−	Tr.	Tr.	Tr.	−	−	−	−	−	−	−
Human serum.	2	−	F.tr.	−	−	−	−	−	−	−	−	−
Horse serum.	2–95			−	−					−	−	Turbid.
	2–99	−	−		−	−	−	−	−			
Pig serum.	3–85	Tr.	±	−	−	Tr.	−	−	−	Tr.	−	+±
Rat serum.	83	−	−	−	−	−	−	±	−	−	−	−
Guinea pig serum.	2–03	−	−	−	−	−	−	−	±	−	−	−
Chicken serum.	50	−	−	++	−	+±	−	−	−	−	−	±
Normal rabbit sera.	4–90	−	−			−	−	−	−			
	4–91	−	−			Tr.	−	−	−			
	4–92	−	−			−	−	−	−			
	4–93	−	−			+	−	−	−			
	4–94	−	−			−	−	−	−			
	5–16			−	−					−	−	−
	5–17			−	−					−	−	−
	5–19			−	−					−	−	±
	5–24			−	−					−	−	−
	5–28									−	−	−

sheep, chicken, Group II human blood. To this group also belong the flocculations with alcoholic extract of horse kidney.

The weak reactions which take place in a rather irregular way may be due to the presence of the same or similar substances in all the extracts, or to the action of certain antibodies on various substances.

In tests with normal rabbit sera only two weak and one moderate reaction occurred, the latter in the case of chicken blood extract. The reacting serum was the only one of the five normal sera tested which gave considerable hemolysis with chicken blood. When normal sera of several other animals and human sera were allowed to act on various blood extracts, the readings being taken after 1 day at 37°C., the tests were mostly negative, except in the case of the horse blood extract. This was flocculated by normal chicken, sheep, and beef sera, the latter reactions being strong. The reaction became weaker, but did not entirely disappear when the beef serum was treated several times with horse blood. After standing 1 more day at room temperature, some other reactions became evident, for instance a strong flocculation of chicken blood extract by beef serum. A flocculation of a methyl alcohol extract of rabbit blood with chicken serum has already been described by Bordet.[2] Pick and Schwarz[3] reported the flocculation of extract of beef serum by normal rabbit serum, and of horse serum extract by the serum of a rabbit injected with this material.

Table II gives the flocculation reactions of a special group of immune sera obtained by injection of a mixture of an alcohol or ether extract of horse blood with protein (diluted pig serum). The preparation of such sera and their properties were described in earlier communications.[1] As stated therein, these sera proved to be specific in their hemolytic action. In the flocculation tests, however, reactions on alcoholic extracts of several bloods were found. It is possible that this lack of specificity was caused by the injection of pig serum. However, no experiments were performed to decide this question.

The two sera prepared with ether extracts of blood alone flocculated only horse blood extract.

[2] Bordet, J., Studies in immunity, New York and London, 1909, 525.
[3] Pick, E. P., and Schwarz, O., *Biochem. Z.*, 1909, xv, 453.

TABLE II.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Alcoholic extract of horse blood + pig serum.	15	±	+	−	−	F.tr.	++	−	−	+	±	+±
	16	−	−	−	−	−	+±	−	−	−	−	Tr.
	20	++	+±	Tr.	F.tr.	++	+±	−	−	−	−	+
	40	−	F.tr.	F.tr.	Tr.	−	+±	−	−	−	−	−
Ether extract of horse blood + pig serum.	4–23	−	−	−	−	±	Tr.	−	−	−	−	−
	4–26	−	−	F.tr.	−	−	Tr.	−	−	−	−	−
	4–28	++	+±	Tr.	−	++±	+++	−	−	Tr.	+	+±
	4–30	−	−	−	−	Tr.	±	−	−	−	−	−
Ether extract of horse blood.	4–38	−	−			−	+±	−	−			
	4–62	−	−			−	++	−	−			

A small number of precipitin sera were tested. Some of them reacted, in most cases on the extract of the homologous blood; but the majority of the reactions were weak (Table I).

According to former results, the property of an immune serum to flocculate seems to run parallel with its content in hemolysins rather than that of agglutinins.[1] In order to see whether the antibodies concerned in the hemolytic and flocculating reactions are identical, we removed the hemolysins and agglutinins in the usual way, by addition of erythrocytes (Table III).

TABLE III.

Immune sera prepared by injection of	Immune serum No.	Absorbed with	Emulsion of extract of horse blood.
Horse blood.	32		+±
" "	32	Horse blood.	−
" "	34		++
" "	34	Horse blood.	+
Alcoholic extract of horse blood + pig serum.	15		++±
" " "	15	Horse blood.	++
" " "	20		++±
" " "	20	Horse blood.	−

The absorption was made by repeated addition of washed blood sediment to the one-half diluted inactivated immune serum (for example, 0.35 cc. of sediment to 0.5 cc. of immune serum in 6 portions, allowing the mixture to stand in the ice box for 1 hour each time until finally no hemolysins or agglutinins could be detected in the super-fluid). The flocculation tests were made as before.

The results suggest that in certain sera a part only of the antibodies producing flocculation are identical with the hemolysins. This view is substantiated by experiments to be described.

Group-Specific Flocculations with Alcoholic Extracts of Human Blood.[4]

It is known that immune sera prepared with human blood of a certain group may contain group-specific agglutinins.[5] As shown in

[4] In cooperation with Dr. Dan H. Witt.

[5] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40. Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419.

a preliminary communication[6] such immune sera for blood II, if sufficiently strong, produce a heavy flocculation with extract of blood of Group II, and act much less strongly upon extracts from Groups I and III. In these comparative experiments the emulsions were prepared by strictly identical technique (Table IV).

It appears from these results that group-specific substances can be extracted from human erythrocytes by means of 95 per cent alcohol. Also absolute alcohol can be used with the same result.

In the paper referred to, no mention was made of the immune sera against Group III blood. With the use of the usual technique, the results were not very distinct, but good flocculations were obtained if the tests were allowed to stand at room temperature for 2 days. However, under these conditions, the flocculations produced by immune serum II with extracts Group II were weaker than usual.

According to these tests (Table IV, *a*), Group III immune sera have the strongest effect upon Group III extracts. A comparison of the phenomena just described with the results of Schiff and Adelsberger[7] will be given below.

Heterologous (Heterogenetic) Reactions.

Monkey Blood Immune Sera-Pig Blood.—In view of the effects, already mentioned, of monkey immune sera on pig blood extract, we made hemolytic and agglutinin tests with these sera on pig blood. The titrations were made as described[1] (Table V).

Fifteen other hemolytic immune sera, including anti-human immune serum, and five normal rabbit sera did not effect complete hemolysis of pig blood in a dilution of 1/50, and most of them were not at all, or only faintly hemolytic. The difference between the monkey blood immune serum and the other immune sera was not pronounced in the agglutinin tests. As the experiments show, the heterogenetic reaction of monkey blood immune serum on pig blood

[6] Landsteiner, K., van der Scheer, J., and Witt, D. H., *Proc. Soc. Exp. Biol.* 1924–25, xxii, 289.

[7] Schiff, F., and Adelsberger, L., *Z. Immunitätsforsch., Orig.*, 1924, xl, 335; *Klin. Woch.*, 1924, iii, 679; *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172. See Hesser, S., *Acta med. Scand.*, 1924, suppl. 9.

TABLE IV.

Read after 20 hours at 37°.

Immune sera prepared by injection of	Immune serum No.	0.2 cc. emulsion of alcoholic extracts of corpuscles.									
		Blood 1 Group I.	Blood 2 Group I.	Blood 3 Group I.	Blood 4 Group I.	Blood 5 Group II.	Blood 6 Group II.	Blood 7 Group II.	Blood 8 Group II.	Blood 9 Group III.	Blood 10 Group III.
Human blood Group II....	21	F.tr.	F.tr.	Tr.	+	+++	+++	+++	+++	F.tr.	0
" " " II....	22	F.tr.	F.tr.	Tr.	+	+++	+++	+++	+++	0	0

TABLE IV*a*.

Read after 2 days at room temperature.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of					
		Blood 11 Group I.	Blood 12 Group I.	Blood 5 Group II.	Blood 14 Group II.	Blood 15 Group III.	Blood 16 Group III.
Human blood Group III.....	4–78	+	+±	+±	+±	++±	++±
" " " III..	4–80	−	−	Tr.	Tr.	+±	±
" " " III	4–81	+	+	+	+	+++	++±

can be demonstrated also by the hemolytic test. The hemolytic titer of the immune sera was rather low, but two of the three baboon blood immune sera had a higher titer for pig blood than for the homologous blood. This heterogenetic reaction is in no way connected with Forssman's heterogenetic antigen, as the immune sera under discussion do not flocculate distinctly extracts of sheep blood or of horse kidney. They have no marked hemolytic action on sheep blood.

Horse Blood Immune Sera-Rat Blood.—A second instance of a heterogenetic reaction brought to light by the flocculation method was

TABLE V.

Bloods.	Titer of agglutinin.			Complete hemolysis in dilution up to			Flocculations with emulsions of alcoholic extracts of	
	Rhesus.	Baboon.	Pig.	Rhesus.	Baboon.	Pig.	Rhesus.	Pig.
Rhesus blood I.S. 4–56.....	800		100	200		50	+±	Tr.
" " " 4–67.....	800		100	200		50	++±	+±
Baboon " " 4-58.....		800	200		<50	100	+±	+
" " " 4–59.....		600	200		50	200		Tr.

TABLE VI.

	Emulsion of extract of rat blood.	Emulsion of extract of horse blood.
Horse blood I.S. 34.....	+++	+±
Same after absorption with rat blood.....	+±	+
" " " " rabbit "	+++	

that of horse blood immune sera on rat blood extract. It was not demonstrable by hemolytic and agglutinin tests. One may infer that an immune serum, acting on a certain substance contained in erythrocytes, need not necessarily have the property of producing hemolysis or agglutination. On the other hand, some observations indicate that if a cellular antigen is divided into fractions, the total of the latter is not so active as the original material. If, for example, blood corpuscles are extracted with alcohol, there is a considerable loss in antigenic and binding properties.

Despite the lack of any visible action on the rat corpuscles, a part of the flocculating antibodies can be absorbed by rat blood (Table VI).

Heterogenetic Antigens of the Forssman Type.—According to the observations of Forssman and other workers along the same lines, a certain type of antigen is present in sheep blood and in cells of very different derivation; as, for example, in those from organs of the horse, guinea pig, etc. Chicken blood corpuscles belong to this group also (Kritschewsky,[8] Hyde[9]). Moreover, Schiff and Adelsberger[7] have described a relationship between human corpuscles Group II and the Forssman antigen.

The tests presented in Table VII give a picture of the relationship of various antigens of the Forssman type. They show that there are certain differences between these various substances.

From a few preliminary experiments, it would seem that dog blood may contain a substance related to the Forssman antigen. The same applies to horse serum, since we have observed reactions of immune sera against sheep blood, chicken blood, and horse kidney on alcoholic extracts of horse serum considerably stronger than those produced by other immune sera.[10]

Differences between the Antigen of Human Blood Group II and the Forssman Antigen.—According to Schiff and Adelsberger, some Forssman immune sera contain an agglutinin against human blood of Group II, and some immune sera prepared with human Group II blood contain hemolysins for sheep blood. This lysin for sheep blood can be absorbed by human corpuscles of Group II, while the sheep lysin of Forssman immune sera is not absorbed by human blood of Group II. We were able to confirm these results. For the above reason the two kinds of antigen are supposed by Schiff and Adelsberger not to be identical. These authors did not obtain distinct flocculation of human blood extracts with human Group II immune sera, but the same sera gave flocculations with alcoholic extracts of Forssman antigen.

It is evident from our findings that the properties of the extracts of human blood Group II are not identical with the reacting substances

[8] Kritchewsky, I. L., *J. Exp. Med.*, 1916, xxiv, 233.

[9] Hyde, R. R., *Am. J. Hyg.*, 1925, v, 217.

[10] See Hanganutziu, M., *Compt. rend. Soc. biol.*, 1924, xci, 1457.

contained in extracts of horse kidney and chicken blood. Furthermore, the reactions of Group II substance and sheep blood extract do not run parallel (Table VII).

When our emulsions were prepared according to the technique of the Sachs-Georgi reaction (without the use of cholesterol) we found positive reactions of human Group II immune sera on extracts of Forssman antigen (horse kidney), but they were distinctly weaker than those with human Group II extracts. The difference between the

TABLE VII.

In this table some results of Table I and additional tests are summarized.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of				
		Human blood Group II.	Horse kidney.	Sheep blood.	Chicken blood.	Dog blood.
Human blood Group II	20	+±	−	+	−	Tr.
" " " II	21	+++	−	+±	−	F.tr.
" " " II	22	+++	−	++±	−	F.tr.
Horse kidney	54	+	++±	+++	++	++
" " *	4-02	−	++±	+++	++	
" "	4-04	−	±	+++	++	±
Sheep blood	11	+++	+++	+++	++	
" "	4-97	−	++±	+++	+±	+±
" "	4-99	±	++±	+++	++	++
Chicken "	41	−	±	+++	++±	+±
" "	43	−	F.tr.	+++	±	F.tr.
" "	3-64	−	±	+++	+±	F.tr.

* In connection with a paper by Trou-Hia-Hsü (Trou-Hia-Hsü, *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 507) it should be mentioned that some of the horse kidney immune sera, for instance No. 4-02, agglutinated sheep blood very distinctly.

Forssman extracts and those of human blood of Group II was still more striking with emulsions prepared in the manner described above. Under these circumstances, the human immune sera did not react on the extract of horse kidney (see Table VII).

In order to study the interrelation between the antigens in question, absorption experiments were carried out. The results are recorded in Table VIII. The tests confirm the difference between the flocculable substances of human blood Group II and sheep blood.

TABLE VIII.

Immune sera prepared by injection of	Emulsions of alcoholic extracts of							Hemolysis in dilutions up to	
	Human blood Group I.	Human blood Group II.	Horse kidney.		Sheep blood.		Chicken blood.		
	2	2	2	1	2	1	1	Sheep blood.	Chicken blood.
Human blood I.S. No. 21	−	+++			+	+++			
" " " " 21 after absorption with sheep blood	−	+±			−	F.tr.			
Human blood I.S. No. 22	−	+++			++	+++			
" " " " 22 after absorption with sheep blood	−	++			−	−			
Sheep blood I.S. No. 4–99			++±	+++	+++	+++	++±	1,600	400
" " " " 4–99 after absorption with human blood Group II			±	+±	+++	+++			
Sheep blood I.S. No. 4–99 after absorption with chicken blood			−	−	−	+±	−	400	<20
Sheep blood I.S. No. 4–99 after absorption with sheep blood			−	−	−	−		<20	

1 = prepared by adding 5 parts of saline, in drops, to 1 part of alcoholic extract.

2 = prepared by blowing 1 part of the alcoholic extract into 5 parts of saline. This emulsion is more stable than 1.

The hemolytic tests were made as above, with 1 drop of 50 per cent blood.

DISCUSSION.

In preceding papers,[1,11] the concept was set forth that the antigens of red cells consist of proteins which are combined with substances of a different chemical nature[12] not in themselves antigenic, or only weakly so, once they become separated from the complex antigen.

The specificity of proteins in general follows the arrangement of the zoological system, as was shown by the comprehensive experiments of Nuttall on serum proteins, and by the important investigations on hemoglobin, by Reichert and Brown. These authors were able to demonstrate that the relationship of the crystal form of hemoglobin parallels to a large extent the zoological relationship of the animals from which the hemoglobin is prepared. The antigens responsible for lysin and agglutinin reactions behave in a different way in that they display sharp differences in related, or even in the same species, and similarities in distant ones. Thus they are not in close correlation with the zoological scale. Examples supporting this view have been presented previously[11] (substances differentiating individuals of one species, Forssman's antigen, the results of absorption of normal and immune sera with blood of closely related species, the results of the isolation of agglutinin fractions from normal serum[13]). It should be emphasized again that after absorbing a normal serum with some kind of blood corpuscles and separating the absorbed agglutinins from the cells, the resulting agglutinin solutions regularly react upon red cells of various species, thus indicating the widespread existence of heterogenetic antigens.[13] Another fact to be mentioned in this connection, is the presence of factors similar to the human isoagglutinogens A and B in animals (von Dungern and Hirschfeld;[14]

[11] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91; Landsteiner, K., and Simms, S., 1923, xxxviii, 127.

[12] In experiments performed with Dr. P. A. Levene, very active preparations of the binding substance (haptene) of Forssman's antigen, made from horse kidney, were found to give no biuret reaction.

[13] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905; *Z. Hyg. u. Infectionskrankh.*, 1907–08, lviii, 213; Oppenheimer, C., Handbuch der Biochemie, Jena, 1910, ii, pt. 1, 410.

[14] von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526.

Landsteiner and Miller[15]).[16]

Our present work furnishes new instances of the existence of similar substances in blood of distantly related species, which can be extracted from the blood by alcohol. The flocculation method has proved of value for the detection of new cases of so called heterogenetic antigens. In one of them the relationship could be shown only by the flocculation reactions; in a second one it was also made evident by hemolytic tests. In view of all these observations the occurrence of so called heterogenetic antigens must be regarded as the rule rather than as the exception.

The assumption that there are present in erythrocytes of different species substances which are similar or identical would seem not to be in keeping with the fact that in most cases immune sera against erythrocytes are specific for the species. Yet it has been shown that an immune serum may not be markedly hemolytic for a certain blood and still contain an antibody active upon a fraction thereof, as in the case described of the flocculation of horse immune sera on rat blood extracts. Furthermore, reactions with high concentrations of immune serum are usually not taken into account, and erythrocytes of only a limited number of species have thus far been examined.

Since the whole corpuscles are much more apt to incite antibody production than are extracts, the proteins of the stromata must play an important part in the process of immunization. Consequently it is rather probable that the specificity of the anti-erythrocyte sera is influenced by the species-specific protein fraction.[17]

In the attempt to express the foregoing concept one may symbolize the serological structure of erythrocytes (and presumably of other animal cells) somewhat as follows:

[15] Landsteiner, K., and Miller, C. P., Jr., *Science*, 1925, lxi, 492.

[16] Chicken immune sera against human blood of Group III contain strong agglutinins for the blood of several animals, as those of the dog, guinea pig, pig, etc. (experiments made in cooperation with Dr. C. P. Miller, Jr., to be published later).

[17] See Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 73, 81.

A, B, C, etc., represent chemical substances combined with the protein (P) or groups contained in such substances. The symbols P_m and P_n indicate proteins that are nearly identical chemically and serologically. P_r is a dissimilar protein of a distant species. According to such a scheme most of the factors (A, B, etc.) are the same in closely related species (m, n), only a few being dissimilar, while in general the factors of distant species (m, r) are different, although identical ones may occur. This view is supported by the fact that when agglutinin immune sera are absorbed with heterologous blood more of the active substances are taken out by cells of a kindred species than by those of others. Also the individual differences within one species can be accounted for by variations of the factors A, B, etc. An idea which may be alternative or supplementary to the first is suggested by the fact that an antibody may react upon several substances not identical, but chemically similar.[18] Accordingly, the occurrence of a certain reaction need not necessarily imply the existence of a special serological factor in every case. Such a view would not require the assumption of a great number of substances in one kind of cells. Which of the two conceptions approaches the facts more closely can be a subject of further studies. The possibility that the reacting substances may be present in different amounts has also to be considered.

The meaning accorded to the specific factors, A, B, C, etc., differs from that embodied in the receptor theory in that the letters signify non-protein chemical substances (or groups of such a substance), separable from the complex antigen, and either not antigenic by themselves or only slightly so (haptenes). If the antigen is broken up, as by the action of alcohol, its antigenic power is greatly impaired. According to our conception, which is at variance with the current opinion, species specificity of cells is of a different order as opposed to species specificity of proteins.

SUMMARY.

1. Flocculation reactions of anti-erythrocyte sera on emulsions of alcoholic extract of blood are described.

[18] *Biochem. Z.*, 1918, lxxxvi, 343.

2. The reactions are markedly species-specific.

3. Besides the homologous reactions certain others—"heterogenetic" ones—have been observed, and in this way the existence of new examples of heterogenetic antibodies has been demonstrated.

4. Group-specific substances can be extracted from human erythrocytes with alcohol and demonstrated by flocculation with group-specific immune sera.

5. A conception of the structure of cellular antigens based upon the known facts, is presented.

LIPOIDS AS THE GROWTH-INHIBITING FACTOR IN SERUM.

By LILLIAN E. BAKER, Ph.D., and ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 20, 1925.)

INTRODUCTION.

Experiments of Carrel and Ebeling have shown that serum has a marked inhibiting action on the growth of fibroblasts *in vitro*, and that this inhibiting action increases with the age of the animal from which the serum is taken.[1] The chemical nature of the inhibiting substance is not known. Carrel and Ebeling have shown[2] that it is not euglobulin or associated with euglobulin, for the CO_2 precipitate from the serum of young animals is somewhat stimulating to the growth of tissues and the filtrate more inhibiting than the original serum. The present work was undertaken to throw light on the chemical nature of the substance. Since experiments on embryonic tissue juice have indicated that its lipoid is somewhat inhibiting to growth, a study of serum lipoids was undertaken. The lipoids of serum and the protein after extraction of the lipoids were tested for their growth-inhibiting properties, each being compared to the original serum and also to Tyrode solution as control.

The Method of Testing the Serum Protein and Lipoids.

The substances were tested with fibroblasts from a 12 year old strain, the two halves of the same tissue being cultivated, one in the experimental medium and one in the control medium, since it has been

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317, 599; 1922, xxxv, 17, 647; xxxvi, 399; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653; xxxviii, 419; *Compt. rend. Soc. biol.*, 1924, xc, 170, 172. Carrel, A., *Compt. rend. Soc. biol.*, 1924, xc, 1005.

demonstrated[3] that the two halves of the same culture of an old strain of fibroblasts grow at a uniform rate when the media are uniform. This is not true of embryonic tissue taken directly from an animal.

The technique of Ebeling[3] for measuring the relative growth of the tissues was used, the original area and the area after 48 hours being measured.

The media used were as follows:

For Serum Protein Compared with Serum or Tyrode.

Experimental medium.	Control medium.
1 drop plasma.	1 drop plasma.
1 " serum protein solution.	1 " serum or Tyrode.
1 " " " " containing 5 per cent tissue juice.	1 " " " " containing 5 per cent tissue juice.

The protein concentration of the serum and of the serum protein was determined by the micro Kjeldahl method and the two adjusted to contain the same per cent of protein nitrogen.

For Serum Lipoid Compared with Tyrode Solution or Serum.

Experimental medium.	Control medium.
1 drop plasma.	1 drop plasma.
1 " lipoid suspension.	1 " Tyrode or serum.
1 " " " containing 5 per cent embryonic tissue juice.	1 " " " " containing 5 cent embryonic tissue juice.

Action of Serum Extracted by Ether on the Growth of Fibroblasts.

Experiments were carried out in which the serum was extracted by ether and also by both alcohol and ether, care being taken to alter the protein as little as possible in order to insure that any change in the growth-inhibiting properties of the serum should be due only to the removal of the lipoid.

The method of extracting by ether found to be the most suitable was as follows:

The serum in a layer about 1/2 cm. thick was evaporated very rapidly in a vacuum desiccator over sulfuric acid by means of a Cenco Hyvac pump. All conditions of the evaporation were so adjusted that the serum supercooled and froze instantaneously. The evaporation was continued until the serum became anhydrous. The instantaneous freezing and drying in the frozen state left the protein of the serum in a very finely divided flaky condition admirably suited to

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

extraction. It was then transferred to a Soxhlet extractor and extracted continuously for 48 to 60 hours with absolute ether freshly distilled over sodium. The extracted serum was finally freed from ether by evaporation *in vacuo* and dissolved in water. The per cent of nitrogen was determined; the proper adjustments of protein and salt concentration were made and the pH brought to 7.6, the reaction of the original serum.

The experiments on the growing tissues showed that in all cases the serum extracted with ether was less inhibiting to the growth of fibroblasts than the original serum, averaging 28 per cent less (see Table I), although it still retained inhibiting properties.

TABLE I.

Rate of Growth of Fibroblasts in Serum Extracted with Ether. Serum Used as Control.

Group* No.	Culture No.	Rate of growth in.		Ratio *E/C.*	Remarks.
		Serum (*C*).	Extracted serum (*E*).		
1	1814 A	2.53	3.09	1.22	48 hrs. growth.
2	479 C	5.95	6.7	1.12	48 " "
3	494 C	2.73	4.07	1.49	24 " "
4	"	5.52	8.59	1.55	48 " "
5	1824 A	2.29	2.52	1.10	24 " "
6	"	4.58	5.6	1.22	48 " "
Average				1.28	

* Each group constitutes three or four experiments.

Action of Serum Extracted with Both Alcohol and Ether on the Growth of Fibroblasts.

The serum extracted with ether alone still contained a considerable quantity of lipoid material no matter how long the extraction continued or how the serum was dried, whether alone or on absorbent material. A subsequent extraction with alcohol always removed a large quantity of lipoid which, after evaporation of the alcohol, was soluble in ether and of quite different character from the material extracted by ether alone. This was also true when the serum was continuously extracted without drying in an apparatus designed for

the ether extraction of liquids. Extraction with both alcohol and ether was therefore undertaken, the serum, ether, and alcohol all being kept at about −10°C. during the entire process. This is a modification of the method used by Young[4] in preparing crystalline serum albumin.

The serum at zero degrees was poured drop by drop with constant stirring into 3 times its volume of 95 per cent alcohol at −10°C., the alcohol being surrounded by a freezing mixture. Only alcohol freshly distilled over KOH to remove aldehydes was used. The mixture was allowed to stand from 1 to 3 hours at a temperature of from −10° to −14°C. It was then packed in a freezing mixture and centrifuged in a centrifuge cooled to 0°C. by circulating brine. The precipitate was washed four times with absolute alcohol, twice with a mixture of absolute alcohol and absolute ether, freshly distilled over sodium, and finally three times with absolute ether. Then after drying in a vacuum the protein was ground to a powder and dissolved in Tyrode solution. If the process had been carefully carried out without rise of temperature until the protein was anhydrous the resulting product was a fine white powder readily soluble in Tyrode solution in a concentration equal to that of the original serum. A very small quantity of the protein remained insoluble but this was negligible in comparison with the whole. The protein concentration was determined by the micro Kjeldahl method and adjusted to be equal to that of the original serum.

The protein so prepared, when tested for growth-inhibiting properties, was found to be much less inhibiting than the original serum, the cultures in the lipoid-free protein growing 78 per cent larger than in the serum (see Table II). These preparations were also less inhibiting than the serum extracted with ether alone in which the cultures grew 28 per cent larger than in the control serum.

It is evident that the removal of lipoid from serum decreases its inhibiting action to a very large extent. In order to ascertain whether the inhibiting action was entirely eliminated, the extracted sera were tested on growing fibroblasts, with Tyrode solution as the control (see Table III). In all cases there was found to be some inhibiting action, the growth averaging 19 per cent less in the serum protein solution. In order to learn whether this was due to the protein itself or perhaps to a small quantity of lipoid contained in it, several preparations of protein were made in which, after the final washing with anhy-

[4] Young, E. G., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 15.

drous ether at −10°C., the protein was transferred to a Soxhlet extractor and extracted for 48 to 60 hours with anhydrous ether (see Table III). This treatment did not lessen the inhibiting effect. One of these preparations seemed to have no inhibiting action in the first passage but the inhibiting effect was quite marked in the second passage and the average of all results even in the first passage gave a figure almost exactly corresponding to that for the protein extracted only at low temperature. It would seem, therefore, that the protein

TABLE II.

Rate of Growth of Fibroblasts in a Solution of Serum Protein Which Had Been Extracted by Alcohol and Ether.
Serum Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*	Remarks.
			Serum (*C*).	Serum protein (*E*).		
1	2211 A	x — 105	4.57	8.54	1.87	
2	2275 A	x — 110	3.82	7.77	2.02	
3	1254 C	x — 119	3.74	6.6	1.76	
4	1273 C	x — 119	3.6	6.03	1.67	2nd passage.
5	1253 C	x — 120	4.36	6.9	1.58	
Average					1.78	

* Each group constitutes three or four experiments.

of the serum has some retarding action on growth, but it is not as marked as the retarding effect of the lipoid. The possibility still remains that a small amount of lipoid is chemically combined with the protein. If so, it is exceedingly small in amount. Reprecipitation of the protein in alcohol and ether has not extracted any measurable quantity.

One preparation (see Table IV) gave results differing from the others. Instead of being inhibiting it had a very slight stimulating action. So far it has not been possible to duplicate this preparation, although exactly the same process has been carried out in the attempt to do so. The explanation probably lies in the fact that the serum of young animals contains a growth-stimulating substance precipitated with euglobulin by CO_2. Experiments on embryonic tissue juice have

shown that its growth-stimulating substance is precipitated by CO_2 but is very unstable and easily destroyed. Probably in this one preparation the growth-stimulating substance escaped complete

TABLE III.

Rate of Growth of Fibroblasts in a Solution of Serum Protein Which Had Been Extracted with Alcohol and Ether.
Tyrode Solution Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*	Remarks.
			Tyrode (*C*).	Serum protein (*E*).		
1	1433 C	x — 110	7.53	5.9	0.78	Serum kept at −10° during extraction.
2	1434 C	x — 120	13.9	11.2	0.81	
3	1388 C	x — 140	9.6	8.8	0.92	1st passage.
4	7797 D	x — 145	6.37	5.49	0.86	
Average of 1st passage in serum extracted only at low temperature.....					**0.84**	
5	1498 C	x — 150	9.4	7.9	0.84	Serum extracted at −10° and also by ether at 37°C. for 48 hrs. in Soxhlet extractor.
6	2430 A	x — 150	12.5	8.4	0.67	
7	7846 D	x — 158	6.18	6.35	1.03	
8	2494 A	x — 163	10.50	8.43	0.80	1st passage.
9	1631 C	x — 163	5.03	4.21	0.83	
Average of 1st passage in serum protein extracted with ether at 37°C. after extraction at −10°C.....					**0.83**	
10	1406 C	x — 140	4.92	3.49	0.71	2nd passage.
11	1515 C	x — 150	9.5	6.27	0.66	
12	2441 A	x — 150	9.8	6.3	0.64	
13	7839 D	x — 158	12.97	8.86	0.68	
14	7860 D	x — 158	5.20	4.06	0.78	
Average of 2nd passage.....					**0.70**	

* Each group constitutes three or four experiments.

destruction while in the other preparations it disappeared, or it may be that this particular serum contained more of the growth-stimulating substance than the others. Experiments are now being carried on to determine whether any growth-stimulating substance can be obtained in the CO_2-precipitated fraction of the extracted sera.

The question of whether the protein is altered by such a strenuous treatment as that used in these experiments is not easy to answer. All evidence which has been obtained so far, however, indicates that there is no alteration except in a very small portion that becomes insoluble. Practically all the protein goes back into solution readily and remains in solution permanently. The protein also contains, as shown by chemical precipitation tests, euglobulin, pseudoglobulins I and II, and albumin, each of which is precipitated as usual and redissolves readily. When plasma is used for the experiment even the fibrinogen is unchanged by the treatment and coagulates on adding a

TABLE IV.

Stimulating Action of Serum Protein Extracted by Alcohol and Ether.

Group* No.	Culture No.	Rate of growth in.		Ratio *E/C.*	Remarks.
		Tyrode (*C*).	Serum protein (*E*).		
1	2246 A	6.2	6.5	1.05	1st passage.
2	2254 A	6.25	7.7	1.23	2nd "
3	2264 A	3.57	4.39	1.23	3rd "
4	1552 C	7.3	9.2	1.26	1st "
5	1574 C	5.9	6.6	1.12	2nd "
6	1589 C	10.9	10.4	0.95	3rd "

* Each group constitutes three or four experiments.

little embryonic tissue juice. Moreover, crystalline serum albumin has been made from the protein of horse serum after this treatment and it is the same in properties as that crystallized from the whole serum.[4] All of these facts point to the conclusion that the protein is not altered.

Further evidence that the protein is unchanged is obtained from experiments on the surface tension of the lipoid-free serum and the normal serum. Du Noüy[5] has shown that a notable change in the time-drop of the surface tension of serum occurs at certain high dilutions, at which a monolayer of the serum molecules is formed. Experiments on the surface tension of the serum protein solutions gave curves at these high dilutions (1:9,000 to 1:12,000) practically duplicating those of the normal serum. Such a concordance of results could

[5] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 547; 1924, xxxix, 37.

hardly be obtained if the protein of the serum were altered chemically or physically.

As a further test immunization experiments were made on rabbits, using as antigens whole chicken serum and chicken serum protein prepared as above. The serum of the rabbits immunized with the serum protein reacted in the precipitin test to the whole serum and to the serum protein with equal intensity, no difference being observable in the quantity of precipitate obtained when the antigens were diluted from 1 in 5 parts to 1 in 2,000 parts. The serum of the rabbit immunized with the whole serum reacted to both antigens, the reaction to the extracted protein being but very slightly less than to the whole serum even at a dilution of 1 in 2,000. Such closely agreeing quantitative results show that no marked change had occurred in the configuration of the protein molecule.

From all the above evidence it seems safe to conclude that the decrease in growth-inhibiting action of the serum can be attributed to the removal of the lipoid.

Action of the Lipoid of Serum on the Growth of Fibroblasts.

The above results which indicate that in removing the lipoid from serum one removes the growth-inhibiting factor are confirmed by tests on the lipoid itself. The problem of obtaining a solution of the lipoid in such a state that it will be utilized by the tissues is difficult. Obviously after once removing it from the serum where in all probability it is held in a chemical or physical union with protein, it cannot be again distributed through an aqueous solution in as suitable a condition for utilization. In the attempt so to do it is important that the lipoid should be kept chemically unaltered if possible. The following technique was chosen as the most suitable for the purpose.

The alcohol which was used for the precipitation and also the first washing, was filtered and evaporated to dryness in a vacuum desiccator at room temperature. It was found best to leave the residue of lipoid dry and in vacuum until just before it was to be used on the cultures of fibroblasts. It was then taken up in a volume of water slightly less than that of the original serum. This was done by flowing the water over the surface of the dry lipoid and rocking the dish gently back and forth. In this way the lipoid and salts were taken up in a uniform emulsion which was very finely divided. If the lipoid was scraped from the dish, such an

even emulsification did not occur and the inhibiting effect on the fibroblasts was not so marked. A sample of the emulsion was tested for its salt concentration by determining the freezing point and enough salt or water added to the remainder to make it isotonic. Also the volume was finally adjusted so that the percentage of lipoid would equal that in the serum from which it was taken.

The lipoid so prepared proved inhibiting to the growth of fibroblasts in comparison with Tyrode solution used as control (see Table V).

TABLE V.

Rate of Growth of Fibroblasts in a Serum Lipoid Emulsion. Tyrode Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*
			Tyrode (*C*).	Lipoid (*E*).	
1	2223 A	x — 106	7.62	4.1	0.54
2	1407 C	x — 106	8.66	7.53	0.87
3	2326 A	x — 128	7.7	6.6	0.86
4	1389 C	x — 137	8.0	3.65	0.46
5	2374 A	x — 141	12.3	6.4	0.52
6	2403 A	x — 141	8.4	4.65	0.55
7	7803 D	x — 149	7.39	6.44	0.87
8	1486 C	x — 147	11.65	9.54	0.82
9	2423 A	x — 147	8.5	5.2	0.61
10	7834 D	x — 157	6.7	6.37	0.95
11	7829 D	x — 157	6.88	4.38	0.63
Average....					0.70

* Each group constitutes three or four experiments.

Not only was the area of growth much less than in Tyrode, averaging 70 per cent, but the cells became fatty and the tissue died after a short time, the lipoid acting as a toxic substance.

For comparing the lipoid with the serum, not only the alcohol used for the first washing but all the wash alcohol and ether were mixed and evaporated. The residue was prepared as above. These total lipoids proved to be 20 per cent more inhibiting than the original serum and were also toxic (see Table VI). The fact that the lipoid was more inhibiting to growth than the original serum would not necessarily mean that it contained all the inhibiting substance of the serum.

As we have seen above, the protein left was still somewhat inhibiting. The smaller growth in the lipoid fraction is probably due to the fact that it has different properties, after its union with the protein of the serum is broken, than it has in its original combination. Also the emulsion of lipoid, although originally adjusted to the same pH as the serum, possesses less buffer action than serum to changes of pH produced by the growth of the tissues.

The question naturally arises as to whether the incease of the inhibiting action of serum with the age of the animal is due to a change in the quantity or character of the lipoid of the serum. A large amount of work will be necessary to answer this question. One preliminary

TABLE VI.

Rate of Growth of Fibroblasts in an Emulsion of Lipoid from Serum. Serum Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*
			Serum (*C*).	Lipoid (*E*).	
1	2375 A	x — 141	7.12	5.72	0.80
2	2404 A	x — 141	5.52	5.05	0.91
3	2424 A	x — 147	8.7	7.2	0.83
4	2432 A	x — 152	7.1	5.3	0.74
5	1499 C	x — 152	5.5	4.65	0.84
6	1595 C	x — 162	8.2	6.0	0.73
Average					0.81

* Each group constitutes three or four experiments.

experiment is interesting in its results. The ratio of the growth in the serum of the old animal to that in the serum of a young animal was 0.81. Exactly the same ratio was obtained for the lipoid fractions derived from these sera respectively and also for the two protein fractions free from lipoid.

Since alcohol extracts other substances than lipoid, a few experiments were carried out to ascertain whether it is the lipoid itself or some other alcohol-extractable material that is responsible for the inhibiting action. The alcoholic solution was evaporated to small volume and a large excess of anhydrous ether added (4 or 5 times its

volume). This precipitated most of the salts, amino acids, and substances insoluble in ether. The mixture was then centrifuged, and both the ether-soluble and ether-insoluble fractions tested on the cultures. The lipoid soluble in ether proved to be the inhibiting substance, while the solution of the salts, amino acids, etc., was found to be either inactive or slightly stimulating. Hence it would appear that lipoid or some substance closely bound to lipoid is the inhibiting substance. Further experiments are being carried out on fractionating this lipoid to ascertain whether the inhibiting substance is one particular substance, or whether all the serum lipoids are inhibiting.

TABLE VII.

Rate of Growth of Fibroblasts in Lipoid Emulsions from Various Sources.

Group* No.	Culture No.	Source of lipoid.	Rate of growth in.		Ratio *E/C*.
			Tyrode (*C*).	Lipoid (*E*).	
1	7804 D	Chicken liver.	10.95	7.02	0.64
2	2363 A	" "	3.3	1.98	0.60
3	2347 A	" "	5.3	3.87	0.73
4	2252 A	Egg lecithin.	13.5	6.76	0.50
5	2364 A	Chicken brain.	5.3	3.13	0.59
6	2348 A	" "	4.2	2.8	0.67
7	2845 A	Embryonic tissue extract.	7.2	5.9	0.82
8	2493 A	" " "	4.55	3.4	0.75 (2nd passage).

* Each group constitutes three or four experiments.

That lipoids, as a class, are inhibiting, is suggested by the results of experiments in which lipoids from egg, chicken brain, chicken liver, and chicken embryonic extract were used. These were prepared in a manner similar to the serum lipoid. As is seen from Table VII, all have proved inhibiting to growth. Further experiments should, however, be carried out, involving the use of these substances in many different concentrations.

SUMMARY.

The growth-inhibiting action of serum has been shown to be due largely to the lipoids.

Serum from which the lipoids have been removed is much less inhib-

iting to the growth of fibroblasts *in vitro* than is the original serum, and only slightly more inhibiting than Tyrode solution.

The lipoids extracted from the serum are toxic and more inhibiting to the growth of fibroblasts than the original serum.

Lipoids extracted from chicken brain, chicken liver, egg, and embryonic tissue have likewise an inhibiting action.

EFFECT OF PREPUBERTY CASTRATION ON SUBSEQUENT CANCER IMPLANTATION.*

By JAMES B. MURPHY, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 21, 1925.)

The effect of gonadectomy on the susceptibility of animals to transplanted tumors has remained a disputed subject. Graf[1] could detect no change from the normal rate of growth when a tumor yielding 100 per cent of takes was implanted in castrated males and females; and Rohdenburg, Bullock, and Johnston,[2] in a similar experiment, noted only a shortening of the latent period. On the other hand, Sweet, Carson-White, and Saxon[3] have reported a very considerable increase in susceptibility both as to the number of takes and the growth rate of tumors inoculated into castrated animals. None of these investigators has recorded very completely either the age of the animals or the time between castration and inoculation. Strong,[4] in an attempt to analyze the time factors involved, noted that mice inoculated 5 to 10 days after castration showed an increased resistance. This persisted but to a diminishing extent until the 16th day. On the other hand, when the inoculation was made during the first 5 days after the operation or later than the 16th day, there was no variation from the normal growth rate of the tumor. Later Strong[5] noted, incidental to another experiment, that prepuberty castration seemed to result in an increased resistance during the middle age period. This observation,

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Graf, R., *Centr. allg. Path. u. path. Anat.*, 1909, xx, 783.

[2] Rohdenburg, G. L., Bullock, F. D., and Johnston, P. J., *Arch. Int. Med.*, 1911, vii, 491.

[3] Sweet, J. E., Carson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1913, xv, 181.

[4] Strong, L. C., *J. Exp. Zool.*, 1922, xxxvi, 67.

[5] Strong, L. C., *J. Exp. Med.*, 1924, xxxix, 447.

however, was based on only nine animals, an insufficient number to be of value, except for the fact that the tumor used for inoculation was one which grows almost without exception in all strains of mice into which it has been inoculated. Leo Loeb[6] has recorded a similar finding in regard to the development of spontaneous tumors. Utilizing a family of mice which normally gives a very high tumor rate, he observed that animals castrated before 6 months of age had a very much lower cancer incidence than intact animals of the same family, while removal of the gonads at a later period had slight or no effect.

The action of prepuberty castration on a large group of factors, particularly those controlling the development of secondary sex characters, as well as on general metabolism and the interrelation of the glands of internal secretion is perhaps too complex for even an attempted analysis at the present. However, it would seem of importance to gather as much information as possible on the general reactions of the body to such an operation. From this point of view we have undertaken in the following experiments to clear up some of the points as to the effect of castration on the resistance to a transplantable tumor.

Prepuberty Castration and Resistance to Tumor Transplants.

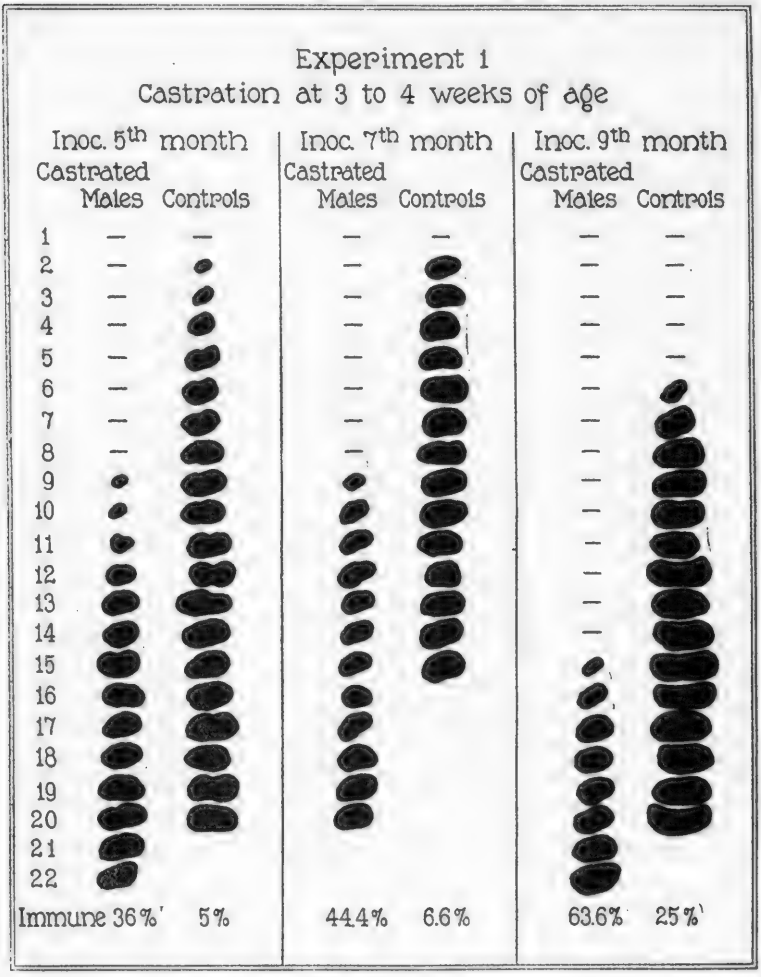
The first test was designed to determine the effect of castration performed during the first few weeks of life on the resistance to transplantable cancer inoculated at later periods.

Operative Technique.—The animals were etherized, the field of operation shaved in the older animals, and the skin cleansed with alcohol. For castration of the males, an incision was made on either side of the scrotum, the testicle drawn out and a ligature tied so as to include the spermatic cord and blood vessels. The testicle was then severed from its connections and the incision closed with silk sutures and painted over with a thin layer of collodion. For the females a small incision was made in either flank in the ovarian region. The ovary, usually found lying in a bed of fat, was gently pulled through the opening, a ligature tied around the vessels, and then the organ was severed from its connections. There was practically no loss of blood in either of these procedures and only an occasional animal, even among the 3 week old mice, died as the result of the operation.

---

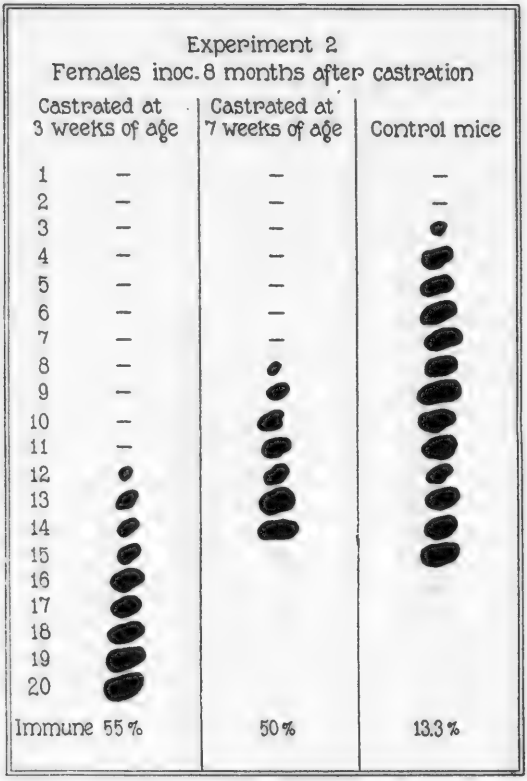
[6] Loeb, L., *J. Med. Research*, 1919, xl, 477; *J. Cancer Research*, 1924, viii, 274. Lathrop, A. E. C., and Loeb, L., *J. Cancer Research*, 1916, i, 1.

Experiment 1.—This group consisted of 64 male mice castrated at from 3 to 4 weeks of age. These were inoculated with Bashford Tumor No. 63 in groups at 4, 6, and 8 months after the operation. The method employed, as in the experiments which follow, was the usual one of introducing a small bit of the tumor material into the subcutaneous tissue of the groin. In each case a suitable number of controls of about the same age were inoculated with the same tumor. The results are shown in Text-fig. 1.



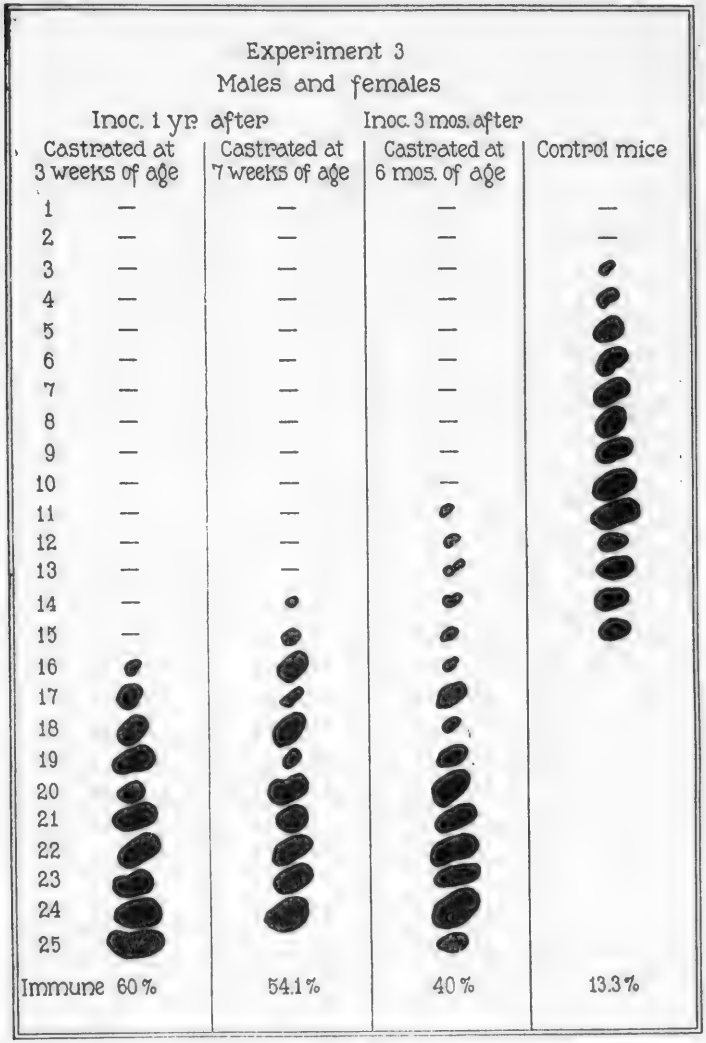
TEXT-FIG. 1.

Experiment 2.—Thirty-four females were castrated between 3 and 7 weeks of age and inoculated 8 months later with the Bashford tumor. For controls fifteen mice of about the same age were inoculated with the same tumor. The results are shown in Text-fig. 2.



TEXT-FIG. 2.

Experiment 3.—In this test there were 49 males and females castrated at 3 and 7 weeks of age and inoculated 1 year later. Another group of twenty-five mice castrated at 6 months of age was inoculated 3 months later. The control group of fifteen mice of about the same age was inoculated with the same tumor. The results are shown in Text-fig. 3.



TEXT-FIG. 3.

Experiment 4.—Eighteen mice, male and female, were castrated at 7 months of age and inoculated with a group of control animals 1 week after the operation. The castrated animals gave 83.4 per cent takes and the controls 88.9 per cent, a difference which would be well within the normal variation of the tumor.

The figures from these four experiments show conclusively that prepuberty castration results in a definite increased resistance of the mice to tumors implanted later in life (Table I). This phenomenon is evident at 3 months after the operation but is more pronounced at the

TABLE I.

Percentage of Immunes.

Experiment No.	Time of inoculation after castration	1 wk.	3 mos.	6 mos.	8 mos.	1 yr.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	Males castrated at 3–4 wks. of age		36.0	44.4	63.6	
	Controls		5.0	6.6	25.0	
2	Females castrated at 3 wks. of age				55.5	
	" " " 7 " " "				50.0	
	Controls				13.3	
3	Males and females castrated at 3 wks. of age					60.0
	" " " " " 7 " " "					54.1
	" " " " " 6 mos. " "		40.0			
	Controls		13.3			13.3
4	Males and females castrated at 7 mos. of age	16.6				
	Controls	11.1				

8 and 12 month period. Even the animals castrated at 6 months of age and implanted with tumor 3 months later show a distinct increase over the normal refractiveness but this is not true of castrated adults inoculated 1 week after gonadectomy. There seems to be no difference between the reaction of the females to removal of the ovaries and the males to removal of the testicles.

DISCUSSION.

The susceptibility or resistance of certain physical types of man to disease was at one time much discussed in medical text-books but

with the discovery of bacteria and their relationship to infections this older conception was lost sight of. In recent years the study of the glands of internal secretion has revealed that the various physical types within any given race of man are largely dependent on the interaction of this complex system. Not only this, but the reaction to disease is also influenced. For example, individuals with status thymicolymphaticus are highly susceptible to acute infections and resistant to the development of malignant disease.[7] Recently Draper[8] has collected considerable material correlating physical types with susceptibility to certain other disease conditions.

Prepuberty castration has a profound effect on the development of the individual. The variations from the normal are not confined to the more or less superficial secondary sex characters but extend even to the development of the bony structures. It should not be surprising that such altered individuals show variations from the normal in their response to disease processes. Such a variation is evident in the increased resistance to transplanted cancer and the lessened incident of the spontaneous disease exhibited by mice castrated before puberty or in early adult life.

SUMMARY.

Male and female mice castrated during the first 7 weeks of life and implanted with cancer at later periods show a resistance definitely higher than do intact animals of the same age. This increased refractiveness is evident at 3 months after the operation but is more pronounced at 8 months to a year. Even castration in early adult life seems to increase the refractory state to later cancer inoculation. On the other hand, adult mice inoculated within a week after castration show slight if any evidence of increased resistance.

[7] Bartel, J., Status thymicolymphaticus und Status hypoplasticus, Leipsic and Vienna, 1912.

[8] Draper, G., Human constitution, Philadelphia, 1924.

EFFECTS OF OBSCURE LESIONS ON ORGAN WEIGHTS OF APPARENTLY NORMAL RABBITS.

By WADE H. BROWN, M.D., LOUISE PEARCE, M.D., and CHESTER M. VAN ALLEN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 3, 1925.)

The results of a series of weight determinations on a group of 350 male rabbits have been reported in a previous paper (1). All of these animals were in apparently good health and free from any external evidence of disease, but on postmortem examination it was found that many of them showed active or healed lesions of some kind. The presence of unsuspected lesions places these latter animals in a class intermediate between those that are normal clinically and pathologically and animals that show obvious signs and symptoms of disease.

The frequency with which gross lesions of various kinds may be encountered in apparently normal rabbits, and the effects of obscure disease processes on the weights of organs are subjects of considerable importance, and the purpose of the present paper is to report briefly the conditions that were found and the results of a comparison of organ weights in those animals of the group that were normal clinically and pathologically and those that showed gross lesions at autopsy.

Methods and Material.

A detailed description of the material on which this report is based and of the methods employed will be found in the first report (1).

The presentation of data bearing on weight relationships will follow the form used in the previous paper with the omission of distribution curves, as the number of animals in some of the subgroups is too small to justify the use of distribution curves.

Classification of Animals and Incidence of Lesions.

On the basis of postmortem findings, the rabbits of this series (350) may be divided into four groups: (1) animals showing no gross lesions

or other evidence of disease, (2) those with few or very slight lesions affecting not more than one organ or tissue, (3) animals with slight lesions of several organs or moderate lesions of one or more organs, (4) animals with marked lesions of one or more organs. For convenience, these groups may be designated as normal or absolute normal, slight, moderate, and marked, or Groups 1, 2, 3, and 4.

The distribution of the 350 rabbits was as follows:

Group.	No. of rabbits.	Per cent.
1	207	59.1
2	71	20.3
3	36	10.3
4	36	10.3
Total	350	100.0

Nature of Lesions.

A detailed description of the lesions noted and their distribution among the four groups of animals will not be given at this time. It seems sufficient to say that the conditions encountered with greatest frequency were:

Coccidiosis of the liver, active or healed	80	animals.
Infection or scarring of the kidneys	27	"
Purulent inflammation of the cranial sinuses	21	"
Otitis media, mastoiditis, or both	11	"
Lesions of the aorta*	16	"
Coccidial cysts in the abdominal cavity not associated with visible lesions of the liver	8	"
Pronounced verminous infection of intestinal tract	7	"
Focal peritonitis of undetermined etiology	4	"

* The majority of the lesions of the aorta occurred in the first 100 rabbits and most of them were comparatively slight degenerations. Subsequently only marked lesions were recorded.

Other conditions that were noted include focal infection of the lungs verminous infection of the liver, small encapsulated abscesses in the subcutaneous tissues and in the deeper tissues of the neck, coccidial cysts in the subcutaneous tissues of the umbilical region, cysts of the thyroid and parathyroids (several cases), hydrocephalus, healed fractures, and adenoma of the left kidney.

With the exception of the sinus and middle ear infections, the great majority of the lesions were either inactive or healed and in none of these animals was the infection sufficiently active to cause a nasal discharge or disturbance of coordination. For example, among the 80 rabbits with coccidiosis of the liver, there were only 20 with fresh lesions; 10 of these were slight (2 or 3 lesions seen), 6 moderate, and 4 marked. Scarring was the only condition noted in 56 cases. In 44 of these the lesions were slight, in 8 moderate, and in 4 marked. In the case of the kidneys the difference was even greater. Scarring was the principle lesion in 25 of the 27 rabbits, while 2 showed a definite acute nephritis, one slight and the other marked. Among the animals with scarred kidneys, there were 9 in which the lesions were classed as slight, 10 moderate, and 6 marked.

The three conditions responsible for nearly all lesions classed as moderate or marked were coccidiosis of the liver, renal abnormalities, and infections of the cranial sinuses, middle ear, and mastoids.

Results of Weight Determinations.

The results of the weight determinations for the entire series of rabbits and for each of the four subgroups are given in Table I. The combined results come first, with the subgroups following in regular numerical order; the values for actual weights precede those for weights per kilo of net body weight (relative weight). In comparing actual weights of organs and coefficients of variation, one should note the order of magnitude of mean body weights and of the coefficients of variation for body weight as these factors affect the actual weights and the coefficients of those organs whose weights are most affected by body weight (1).

DISCUSSION.

In considering the results recorded in Table I the first point to which attention may be directed is the frequency with which active or heaed lesions were encountered. The incidence of gross lesions in this series of rabbits was almost exactly 40 per cent; from what is known concerning the occurrence of microscopic lesions in such organs as the brain (2, 3) and heart (4), it is not unlikely that a microscopic

TABLE I.

Results Obtained for Body and Organ Weights According to Groups.

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Gross body weight.		350	2,240.3	2,225	1,400	3,500	352.62	237.84	15.74
	1	207	2,293.9	2,250	1,575	3,450	358.64	241.90	15.63
	2	71	2,227.2	2,200	1,725	3,500	283.07	190.93	12.71
	3	36	2,319.7	2,225	1,550	3,375	393.03	265.10	16.94
	4	36	2,166.0	2,050	1,400	3,100	357.24	240.96	16.50
Gastrointestinal mass.		350	416.35	410	115	710	80.89	54.53	19.42
	1	207	402.07	415	235	710	80.58	54.35	20.04
	2	71	428.40	420	295	690	66.00	44.52	15.41
	3	36	406.60	392.5	305	640	68.90	46.47	16.94
	4	36	378.60	382	115	540	83.30	56.19	22.00
Net body weight.		350	1,848.5	1,800	1,150	3,010	323.27	218.04	17.49
	1	207	1,868.5	1,830	1,275	2,950	324.07	218.59	17.34
	2	71	1,793.9	1,800	1,215	3,010	274.49	185.14	15.30
	3	36	1,909.3	1,807	1,150	2,950	382.70	258.13	20.04
	4	36	1,779.0	1,695	1,175	2,685	321.86	216.09	18.09
Heart.		348	5.26	5.12	3.2	10.18	0.96	0.64	18.23
Actual.	1	206	5.23	5.13	3.2	9.50	0.98	0.66	18.74
	2	71	5.14	5.00	3.6	7.68	0.74	0.50	14.05
	3	36	5.35	5.20	3.75	7.70	0.87	0.57	16.26
	4	35	5.13	5.21	3.55	10.18	1.28	0.86	24.97

Relative.		348	2.85	2.81	1.95	4.42	0.35	0.24	12.32
	1	206	2.81	2.76	1.95	4.21	0.32	0.22	11.38
	2	71	2.94	2.86	2.27	3.75	0.32	0.22	10.81
	3	36	2.84	2.86	2.06	3.60	0.35	0.24	12.32
	4	35	2.89	3.00	2.21	4.42	0.43	0.29	14.74
Liver.		350	84.30	80.00	50.00	150.00	19.72	13.30	23.39
Actual.	1	207	85.85	82.00	52.00	150.00	20.59	13.89	23.98
	2	71	82.10	80.00	50.00	135.00	17.49	11.80	21.30
	3	36	80.82	79.00	52.50	120.00	16.49	11.12	20.40
	4	36	83.56	83.00	50.00	140.00	20.88	14.08	24.98
Relative.		350	46.46	44.90	23.20	97.40	11.84	7.99	25.48
	1	207	48.83	44.60	23.20	80.60	11.19	7.55	22.92
	2	71	46.40	47.20	27.80	75.80	10.41	7.02	22.43
	3	36	44.21	39.40	26.00	97.40	14.76	9.95	33.40
	4	36	47.95	45.35	28.10	85.70	13.75	8.57	28.67
Kidneys.		348	12.70	12.50	7.15	22.73	2.07	1.40	16.30
Actual.	1	207	12.70	12.55	7.15	20.07	2.12	1.43	16.69
	2	71	12.21	12.22	8.80	16.70	1.98	1.34	16.21
	3	35	12.77	12.86	9.08	22.73	2.49	1.68	19.50
	4	35	13.19	12.70	9.92	20.90	2.29	1.54	17.36
Relative.		348	6.97	6.84	3.45	17.28	1.26	0.85	18.08
	1	207	6.90	6.69	3.45	11.77	1.16	0.78	16.81
	2	71	6.89	6.92	4.89	8.77	0.89	0.60	12.92
	3	35	6.83	6.60	4.55	10.67	1.38	0.93	20.21
	4	35	7.45	7.17	5.49	17.28	2.90	1.96	38.99

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Spleen.		347	0.990	0.90	0.08	3.20	0.436	0.294	44.04
Actual.	1	205	0.972	0.89	0.08	2.40	0.419	0.283	43.10
	2	70	0.919	0.80	0.28	2.20	0.405	0.273	44.07
	3	36	1.065	1.015	0.23	3.20	0.393	0.265	36.90
	4	36	1.156	1.075	0.31	2.40	0.521	0.351	45.07
Relative.		347	0.531	0.49	0.035	1.714	0.214	0.144	40.30
	1	205	0.532	0.482	0.035	1.466	0.236	0.159	44.36
	2	70	0.522	0.476	0.186	1.043	0.207	0.14	39.65
	3	36	0.561	0.526	0.137	1.23	0.221	0.149	39.40
	4	36	0.661	0.631	0.182	1.714	0.299	0.202	45.23
Thymus.		349	2.210	2.13	0.45	5.90	0.848	0.572	38.37
Actual.	1	206	2.321	2.22	0.83	5.22	0.821	0.554	35.37
	2	71	2.165	2.135	0.45	4.70	0.895	0.604	41.34
	3	36	2.058	1.99	0.855	3.40	0.694	0.468	33.72
	4	36	2.119	1.952	1.06	5.90	0.949	0.640	44.78
Relative.		349	1.23	1.147	0.334	2.72	0.451	0.304	36.64
	1	206	1.247	1.192	0.443	2.71	0.430	0.290	34.48
	2	71	1.202	1.198	0.334	2.72	0.473	0.319	39.35
	3	36	1.082	1.086	0.446	2.135	0.417	0.281	38.54
	4	36	1.217	1.045	0.512	2.59	0.505	0.341	41.53
Testicles.		290	4.86	4.85	0.81	9.12	1.63	1.10	33.54
Actual.	1	184	4.74	4.86	0.81	9.12	1.64	1.11	34.60
	2	49	4.72	4.91	2.29	8.98	1.32	0.89	27.75
	3	26	5.60	5.52	2.10	8.65	1.65	1.13	29.46
	4	31	4.70	5.05	2.16	9.02	1.57	1.06	33.40

Relative.		290	2.61	2.62	0.47	4.93	0.70	0.47	26.90
	1	184	2.53	2.67	0.47	4.79	0.738	0.498	29.17
	2	49	2.59	2.59	1.46	4.32	0.607	0.409	23.44
	3	26	2.98	3.02	1.09	4.93	0.75	0.506	25.17
	4	31	2.58	2.70	1.09	4.16	0.747	0.504	28.95
Brain.									
Actual.		150	9.31	9.24	7.42	12.03	0.78	0.526	8.37
	1	84	9.45	9.29	7.52	11.54	0.78	0.526	8.25
	2	30	9.17	9.15	8.05	10.10	0.51	0.344	5.50
	3	15	9.34	9.35	8.47	10.25	0.497	0.335	5.30
	4	21	9.36	9.40	7.42	12.03	1.09	0.735	11.64
Relative.		150	5.113	5.06	3.33	8.16	0.87	0.58	16.92
	1	84	5.001	5.35	3.25	6.90	0.87	0.586	17.40
	2	30	5.30	5.30	4.19	7.04	0.72	0.485	13.40
	3	15	5.15	4.93	3.94	8.16	1.02	0.688	19.80
	4	21	5.23	5.19	3.33	8.16	0.82	0.55	15.68
Thyroid.									
Actual.		349	0.23048	0.185	0.085	1.570	0.16209	0.10933	70.33
	1	206	0.22734	0.185	0.085	1.570	0.16134	0.10882	70.90
	2	71	0.19580	0.180	0.090	0.445	0.07708	0.05199	39.36
	3	36	0.27540	0.207	0.110	0.810	0.17388	0.11728	63.14
	4	36	0.27364	0.185	0.100	0.900	0.21132	0.14254	77.23
Relative.		349	0.12324	0.0975	0.0504	0.7300	0.0757	0.05106	61.47
	1	206	0.12015	0.1033	0.0504	0.7300	0.07071	0.04769	58.85
	2	71	0.10927	0.0976	0.0562	0.2372	0.03357	0.02264	30.72
	3	36	0.1431	0.1138	0.0576	0.3520	0.07633	0.05148	53.34
	4	36	0.14885	0.11095	0.0587	0.4825	0.10287	0.06939	69.11

TABLE I—*Continued.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Parathyroids.		349	0.01256	0.0120	0.002	0.0350	0.00486	0.00328	38.69
Actual.	1	206	0.01258	0.0120	0.005	0.0350	0.00459	0.00310	36.48
	2	71	0.01204	0.0110	0.002	0.0320	0.00489	0.00330	40.75
	3	36	0.01311	0.0120	0.007	0.0280	0.00455	0.00307	34.71
	4	36	0.01322	0.0115	0.005	0.0300	0.00625	0.00422	47.43
Relative.		349	0.00692	0.00631	0.00109	0.02175	0.00278	0.00187	40.13
	1	206	0.00679	0.00631	0.0026	0.02175	0.00290	0.00196	42.71
	2	71	0.00682	0.00619	0.00109	0.01510	0.00265	0.00179	38.85
	3	36	0.00729	0.00623	0.00122	0.01569	0.00298	0.00201	40.88
	4	36	0.00771	0.00710	0.00296	0.01835	0.00375	0.00253	48.64
Hypophysis.		348	0.028	0.028	0.015	0.044	0.00517	0.00349	18.46
Actual.	1	205	0.02795	0.027	0.015	0.044	0.00521	0.00351	18.64
	2	71	0.02421	0.027	0.015	0.042	0.00557	0.00376	23.01
	3	36	0.02947	0.030	0.018	0.040	0.00549	0.003703	18.63
	4	36	0.0287	0.030	0.019	0.038	0.00448	0.00304	15.60
Relative.		348	0.0154	0.0153	0.00728	0.0294	0.0031	0.00209	20.27
	1	205	0.01565	0.1513	0.00728	0.0294	0.00305	0.00206	19.50
	2	71	0.01521	0.0147	0.00808	0.0218	0.00283	0.00191	18.61
	3	36	0.0158	0.01591	0.0095	0.02435	0.00331	0.00223	20.93
	4	36	0.01656	0.01666	0.0095	0.0255	0.00355	0.00239	21.44
Suprarenals.		349	0.38012	0.358	0.115	1.050	0.15501	0.10455	40.78
Actual.	1	207	0.38498	0.355	0.115	1.020	0.15348	0.10352	39.86
	2	70	0.3524	0.3285	0.180	0.830	0.13461	0.0907	38.20
	3	36	0.42539	0.4550	0.180	0.785	0.13907	0.0938	32.70
	4	36	0.4144	0.354	0.155	1.050	0.19407	0.1309	46.83

Relative.		349	0.2088	0.1967	0.0804	0.530	0.07503	0.05061	35.93
	1	207	0.20481	0.1976	0.0804	0.530	0.07591	0.0512	37.06
	2	70	0.1958	0.1816	0.0852	0.5282	0.0681	0.04594	34.78
	3	36	0.2262	0.250	0.1068	0.446	0.0713	0.04809	31.52
	4	36	0.23401	0.1988	0.1023	0.528	0.10137	0.06837	43.31
Pineal.		348	0.01609	0.014	0.005	0.030	0.00429	0.00289	26.66
Actual.	1	206	0.01635	0.0155	0.006	0.030	0.00426	0.00287	26.05
	2	70	0.0157	0.0150	0.005	0.029	0.00397	0.00268	25.28
	3	36	0.01625	0.016	0.008	0.030	0.00471	0.00318	27.13
	4	36	0.01455	0.0145	0.007	0.025	0.00373	0.00252	25.63
Relative.		348	0.00885	0.00868	0.00273	0.01873	0.00245	0.00165	27.68
	1	206	0.0089	0.0086	0.00273	0.01873	0.00243	0.00164	27.31
	2	70	0.00887	0.00864	0.00314	0.0146	0.00229	0.00155	25.82
	3	36	0.009	0.00922	0.00385	0.01617	0.00284	0.00192	31.55
	4	36	0.00834	0.00791	0.00465	0.01285	0.00235	0.00159	28.18
Popliteal lymph nodes.		242	0.25539	0.245	0.085	0.720	0.08241	0.05559	32.27
Actual.	1	160	0.25766	0.247	0.085	0.720	0.07807	0.05266	30.30
	2	41	0.2698	0.270	0.100	0.510	0 0914	0.06165	33.87
	3	17	0.2223	0.205	0.130	0.410	0.07999	0.05395	35.98
	4	24	0.2235	0.227	0.100	0.400	0.0699	0.04715	31.27
Relative.		242	0.13573	0.1361	0.0525	0.382	0.04873	0.03287	35.90
	1	160	0.14021	0.1363	0.0525	0.382	0.04004	0.02700	28.56
	2	41	0.1504	0.1467	0.0568	0.314	0.0582	0.03926	38.69
	3	17	0.1175	0.1086	0.0573	0.261	0.05331	0.03596	45.37
	4	24	0.1287	0.1208	0.0587	0.2341	0.04563	0.03078	35.45
Axillary lymph nodes.		242	0.17585	0.175	0.040	0.400	0.05975	0.0403	33.98
Actual.	1	160	0.17937	0.175	0.040	0.370	0.05934	0.04092	33.08
	2	41	0.1759	0.175	0.055	0.400	0.0771	0.05200	43.83
	3	17	0.15241	0.160	0.085	0.260	0.04572	0.03084	30.00
	4	24	0.1611	0.150	0.060	0.357	0.06598	0.0445	40.95

TABLE I—*Concluded.*

Organ.	Group No.	No. of animals.	Arithmetical mean.	Median.	Minimum.	Maximum.	Standard deviation.	Probable error.	Coefficient of variation.
			gm.	*gm.*	*gm.*	*gm.*	±*gm.*	±*gm.*	*per cent*
Relative.		242	0.09722	0.091	0.01865	0.2425	0.03779	0.02549	38.87
	1	160	0.09843	0.0926	0.01865	0.200	0.03595	0.02425	36.52
	2	41	0.0989	0.097	0.0273	0.2425	0.0414	0.02792	41.86
	3	17	0.0806	0.0787	0.0453	0.1438	0.03043	0.02053	37.75
	4	24	0.09373	0.0813	0.02556	0.2205	0.04403	0.026698	46.97
Deep cervical lymph nodes.		124	0.15579	0.140	0.035	0.405	0.0732	0.04938	46.98
Actual.	1	80	0.15872	0.1425	0.045	0.405	0.07662	0.05168	48.27
	2	23	0.1433	0.140	0.070	0.250	0.0508	0.03426	35.45
	3	9	0.18125	0.140	0.085	0.290	0.06878	0.04639	37.95
	4	12	0.15708	0.130	0.035	0.280	0.06864	0.0463	43.69
Relative.		124	0.08207	0.0724	0.02052	0.211	0.03712	0.02504	45.23
	1	80	0.0821	0.0726	0.0327	0.211	0.03852	0.02598	46.92
	2	23	0.0825	0.0714	0.0358	0.1695	0.03528	0.0238	42.76
	3	9	0.07463	0.06275	0.0470	0.121	0.02658	0.01793	35.61
	4	12	0.08818	0.0842	0.02052	0.1425	0.03402	0.02295	38.58
Mesenteric lymph nodes.		133	3.488	3.32	1.34	9.19	1.31	0.88	37.53
Actual.	1	81	3.66	3.57	1.35	9.19	1.43	0.964	39.07
	2	26	3.40	3.32	1.72	7.10	1.12	0.755	32.94
	3	10	3.26	3.32	1.80	5.87	1.06	0.715	32.51
	4	16	2.91	2.86	1.34	4.60	1.34	0.904	46.05
Relative.		133	1.828	1.763	0.72	3.71	0.642	0.43	35.12
	1	81	1.89	1.847	0.72	3.71	0.684	0.46	36.19
	2	26	1.88	1.76	1.165	3.39	0.534	0.36	28.28
	3	10	1.60	1.507	0.826	2.77	0.555	0.374	34.70
	4	16	1.56	1.61	0.772	2.57	0.505	0.341	32.38

examination of tissues from all organs would show a material increase in the percentage of animals with lesions of some kind.

It is obvious, therefore, that the apparent state of health and the presence or absence of lesions do not run parallel. In this series of 350 rabbits there were 72 rabbits (20 per cent) with moderate or marked lesions, but these animals were apparently as vigorous and healthy as those that were entirely free from any gross evidence of disease. This situation might have been altered by a more prolonged period of observation. This is especially true of those animals with sinus or middle ear infections, acute coccidiosis, and acute nephritis as these conditions are not infrequently accompanied by signs or symptoms of disease. In fact, diseases accompanied by lesions less extensive than many of those seen in animals of this series frequently cause severe symptomatic disturbances or even death. Strictly speaking, therefore, the physical condition of an animal is an index of the effects of disease or of the manner in which an animal reacts to infections of various types rather than of the probable presence or absence of disease processes.

There is, however, a remarkably close numerical relation between the extent of the lesions found in this group of animals and the resistance ordinarily displayed by rabbits. For example, the number of animals found to be normal, or nearly so, (Groups 1 and 2) was 278, while only 72 (Groups 3 and 4) showed any considerable degree of abnormality. That is, approximately 80 per cent (79.42 per cent), or four out of every five rabbits, were practically free from gross evidence of disease, while one in five (20.56 per cent) showed evidence of a serious affection, and only one in ten (Group 4, 10.28 per cent) of a disease of marked severity. These figures agree almost exactly with repeated observations on the resistance of rabbits to various experimentally induced diseases as well as to toxic agents of many kinds. As stated by us (5), the resistance ratio of rabbits, high: intermediate: low, is approximately 1:3:1, while one animal in every ten may be expected to display a resistance of a very low order.

Similar conditions obtain in the case of organ weights. An examination of the data in Table I shows that the differences in the weights of organs of rabbits with no gross lesions, or lesions that were slight, moderate, or marked, are very small and that the figures for the sub-

groups are comparable to those for the entire series; in many instances the agreement is almost absolute and in all cases the values are within the limits of error for any one of the four groups.

Still, one can detect a definite relation between the results obtained and the pathological status of the animal. This may be brought about by a consideration of the relative magnitude of mean weights per kilo of net body weight and of the corresponding coefficients of variation. Thus, the distribution of largest and smallest mean weights and of the largest and smallest coefficients of variation according to groups is as follows (Table II):

TABLE II.

Distribution of Maximum and Minimum Mean Weights and Coefficients of Variation According to Groups.

Organ.	Mean weight.		Coefficient.	
	Maximum.	Minimum.	Maximum.	Minimum.
Thyroid	4	2	4	2
Hypophysis	4	2	4	2
Suprarenals	4	2	4	3
Parathyroids	4	1	4	2
Pineal gland	3	4	3	2
Popliteal lymph nodes	2	3	3	1
Axillary " "	2	3	4	1
Mesenteric " "	1	4	1	2
Thymus	1	3	4	1
Heart	2	1	4	2
Brain	2	1	3	2
Liver	1	3	3	2
Kidneys	4	3	4	2
Spleen	4	2	4	3
Deep cervical lymph nodes	4	3	1	3

From this tabulation, it is at once apparent that there is a close parallelism between the values obtained for the endocrine glands and disease processes. With a single exception, the heaviest organs and the greatest variation in weight occur in animals with the most marked lesions, while the smallest organs and the greatest constancy are found in animals with slight lesions. The one striking exception to this rule

is in the case of that organ of the group the weight of which is probably least reliable; namely, the pineal gland.

The relation between weights and lesions is less obvious in the case of other organs. The coefficients of variation follow the same order as for the endocrine glands but in the case of mean weights the relation appears to be reversed. In most instances, the largest weights are found in animals with few or no lesions (Groups 1 and 2), while the small weights occur in animals with moderate or marked lesions (Groups 3 and 4); in general, the reverse is true for coefficients of variation.

The exceptions to this general tendency are susceptible of various explanations. In some instances, the organs concerned are either the seat of lesions or are directly affected by disease processes. This applies particularly to the liver, the kidneys, the spleen, and the deep cervical lymph nodes. The weights of both the liver and the kidneys may be greatly increased or diminished but the probable end-result is somewhat different in the two cases. Active coccidiosis usually increases the weight of the liver and the same is true for active or acute infections of the kidneys. On the other hand, scarring of the liver from coccidiosis tends to diminish the weight, while in the case of the kidneys the effect of chronic disease is not infrequently in the opposite direction, so that the weights of scarred kidneys may be greatly increased (see maximum and minimum values, Table I). In like manner, the spleen is apt to be enlarged in animals with abdominal disease, while the deep cervical lymph nodes show a similar response to infections of the cranial sinuses.

In the case of the heart and brain, the occurrence of the smallest weights in normal animals is what might be expected of organs that are not known to play an active part in the general reaction to disease. Still, both of these organs conform to the general tendency in other respects and, as a matter of fact, this one discrepancy is more apparent than real as the differences between the mean weights of the heart and brain for animals of Groups 1 and 3 are so small that Group 3 might well be substituted for Group 1 in the column of smallest weights.

Another interesting condition that is brought out by this arrangement of organ weights and coefficients (Table II) is the divergent tendency of superficial and deep lymph nodes. In our experience, these

two groups of lymph nodes rarely behave alike, and this is true in the present instance. The superficial lymph nodes, popliteals and axillaries, are the only organs whose weights were most constant in strictly normal animals, while the deep lymph nodes, mesenterics and deep cervicals, showed the greatest irregularity in the same class of animals. The superficial nodes are extremely sensitive in their reaction to infection, and it is not surprising to find the most constant weights in normal animals, but the deep cervical and mesenteric lymph nodes appear to be influenced more by local than by systemic conditions or by conditions other than those that affect the superficial nodes.

The thymus, mesenteric lymph nodes, and the liver have the distinction of being the only organs with weights largest in normal animals, a finding which may be attributed to conditions affecting alimentation or general nutrition as these three organs are peculiarly susceptible to such influences.

Our object in directing attention to these details of mass relationships is merely to show that, in spite of the small numerical differences between the values obtained for the weights of organs of different groups of animals, there is a distinct suggestion of a correlation between obscure disease processes and the weights of many if not all of the organs of the body. The material on which this study is based is not sufficient to enable one to define the relation in exact terms or even to say with certainty that a definite relation exists. The number of animals in Groups 1 and 2 is probably sufficient to indicate what may be expected in the case of rabbits that are normal or nearly so; the uncertainty is attached to animals with moderate or marked lesions. But more material is now available, and when this has been analyzed we will be able to speak with greater assurance. Meantime, the evidence that has been presented is sufficiently definite to warrant certain tentative conclusions.

In the first place, it would appear that in estimating the weights of organs of large groups of supposedly normal rabbits the results are not materially disturbed by the inclusion of animals with slight or even with moderate lesions and are not greatly affected by animals with marked lesions. In general, it may be said that so long as the health and vigor of the animal remains unimpaired, the presence of

lesions is not associated with quantitative changes in the weights of organs that are sufficiently large to be of any statistical significance.

Possible exception may be made in the case of organs that are themselves the seat of disease, such as the kidneys and liver, and those organs which, by reason of their position or function, are directly affected by the disease processes, as in the case of the spleen and the deep cervical lymph nodes; but, even in these cases, the changes in mean values are relatively small so long as we deal with groups of considerable size. Extreme changes may be encountered in individual animals but changes in one direction are apt to be counterbalanced by those in the opposite direction, so that irregularity or variability becomes the outstanding feature when mean values are compared with those for normal or slightly abnormal animals.

This situation is in striking contrast with conditions that are found when diseases of the same nature have progressed to the point of clinical recognition. It is not surprising, therefore, to find a suggestion of differences in the weights of organs such as are found in cases of outspoken disease.[1] The organs in which such changes are most apparent are not those directly affected by the disease but are organs which are chiefly concerned with processes of animal economy or in the reaction to disease, especially the organs of internal secretion and the lymphoid tissues. The tendency of these organs to undergo changes in weight, which vary according to the lesions present, is strongly suggestive of a functional response on the part of these organs.

In this connection, Group 2, composed of animals with minor lesions, is of especial interest as the animals of this group gave the most consistent results; the weights of organs were either very large or very small but in either case there was less variation in weight than in any other group. This suggests that the presence of some slight lesion or focus of infection may serve as a stimulus and at the same time regulate functional activity in such a way as to maintain the weights of organs at or near the normal level and within limits that are

[1] The results of observations made on a group of animals with clinically recognizable diseases of spontaneous origin will be reported in a subsequent paper. Reference to conditions that are found in such animals are based on the results of this study.

even narrower than those of normal rabbits. This suggestion is strengthened by the reversal of these conditions in animals with more extensive lesions.

CONCLUSIONS.

It would appear that, in rabbits, any departure from the normal gives rise to some change in the mass and mass relationships of organs and the tendency to variation. These changes follow a regular order depending upon the degree of abnormality, but the magnitude of the change is of such an order that it is barely perceptible until the abnormality is sufficiently pronounced to be recognized by signs and symptoms of disease or by physical deterioration. In apparently healthy and vigorous rabbits only those with extensive lesions show differences in the mean weights of organs, when compared with the normal, that are of any material significance from a statistical point of view; but all changes are of importance from the point of view of the reaction to disease.

SUMMARY.

A group of 350 normal rabbits was studied with reference to the occurrence of obscure lesions of various kinds and the probable or possible effect of such lesions or disease processes on organ weights. The results of the investigation are presented in the form of comparative tabulations.

It was found that so long as the animals remained in apparently good health the values obtained for organ weights of animals with lesions did not differ materially from those for animals that were entirely free from lesions. There were, however, slight deviations from the normal which appeared to be significant in that they suggested a functional response similar in character to the more marked changes in mass and mass relationships that occur in rabbits presenting clinical symptoms of disease due to the same causes.

BIBLIOGRAPHY.

1. Brown, W. H., Pearce, L., and Van Allen, C. M., *J. Exp. Med.*, 1925, xlii, 69.
2. McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.
3. Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.
4. Miller, C. P., Jr., *J. Exp. Med.*, 1924, xl, 543.
5. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1923, xxxvii, 799.

THE INFLUENCE OF THE AUTONOMIC NERVOUS SYSTEM ON THE FUNCTION OF THE THYROID GLAND.*

By J. HAMILTON CRAWFORD, M.D., and J. N. J. HARTLEY, F.R.C.S.

(*From the Pharmacology Department of Edinburgh University, Edinburgh, Scotland.*)

Plate 1.

(Received for publication, May 13, 1925.)

The mechanism of the control of the function of the thyroid gland is a subject of great interest both from a physiological point of view and also because of the part it may play in the pathological changes which so frequently take place in this gland. The studies of Andersson (1), Berkley (2), and Rhinehart (3) on the anatomy of the thyroid have shown that nerves pass to the gland from the superior and recurrent laryngeal nerves and also from the cervical sympathetic cord by way of the thyroid arteries. It is still doubtful whether the fibers in the laryngeal nerves are of vagal or sympathetic origin. Within the gland many of the fibers end on the terminal vessels but others are distributed to the walls of the vesicles where they end at the base of the gland cells. The fact that nerve fibers pass to the secretory cells has led to the supposition that the thyroid like other glands, such as the salivary glands, is under nerve control. Attempts have been made to settle this question by histological, physiological, and chemical studies.

Histological Evidence.—Changes in the gland cells supposed to indicate active secretion have been described by some investigators as following the injection of pilocarpine (4, 5) and adrenalin (6), while others obtained negative results in similar experiments (6, 7). Division of the laryngeal nerves (8) or of the cervical sympathetic (9) has been reported to cause degeneration of the gland. This action in the case of the nerves first mentioned has been also attributed to hyperemia (10) or to section of sympathetic fibers (11). Stimulation of the cervical sympathetic from time to time over a period of 1 to 2½ months has been

* The expenses of this research were defrayed by a grant from the Earl of Moray Fund, Edinburgh University.

stated to cause changes indicative of increased activity, while the opposite effect followed excision of the cervical sympathetic (12, 13). Later workers have been unable to confirm this (14). Stimulation of the superior cervical ganglion or injecting *B. bronchisepticus* into it has been reported as producing either thyroid hyperactivity or exhaustion according to the duration of the stimulus (15), and degenerative lesions have been described in the cervical ganglia in cases of exophthalmic goiter (16). No histological difference could be detected between a graft of thyroid in which nerve control was absent and the part of the gland which remained after partial thyroidectomy (17–19).

Physiological Evidence.—Stimulation of the laryngeal nerves or injection of thyroid extract has been described as causing an increase in the activity of the depressor nerve (20), the splanchnic nerve (21), and the vagus (22), and an increased response to injections of adrenalin (20, 23). Other investigators have been unable to confirm these findings (24–26). Stimulation of the cervical sympathetic has been stated to cause an increased response to adrenalin after a latent period of an hour. Previous thyroidectomy prevented the action but adrenalectomy did not alter the response (27). However, an increased excitability of the autonomic nervous system in thyroidectomised animals was found by others to follow repeated injections of a constant dose of adrenalin (28). Cannon and Smith described an increase in rate of the denervated heart after massage of the thyroid or stimulation of the cervical sympathetic, which they considered in both instances was due to the discharge of thyroid secretion (29). A difference of electric potential in the gland has been shown after stimulation of the cervical sympathetic, the injection of adrenalin, or splanchnic stimulation, which was absent when other nerves were stimulated or anemia of the gland produced (30, 31). Cannon, Binger, and Fitz (32) utilising a method of phrenic-cervical sympathetic anastomosis devised by Langley and Anderson (33) reported that this caused symptoms resembling exophthalmic goiter. This work has not since been confirmed by the workers mentioned or by others (34–36). Marine found no change in the functioning capacity of the gland after sectioning the nerves running in the adventitial coats of the vessels and encasing the gland in paraffin (37).

Chemical Evidence.—Removal of the inferior cervical ganglion has been reported to cause a loss of weight and reduction of the iodine content while section of the vagus was without effect (11). Stimulation of the superior thyroid vessels with their accompanying nerves or the vagosympathetic trunk has been said to cause a similar effect (38, 39), but van Dyke could find no greater variations than those normally present (40).

In the present series of experiments the influence on the histology of the thyroid gland of section or stimulation either singly or combined of the various nerves which pass to the thyroid gland has been investigated. As a preliminary we studied the histology of the thyroid in normal animals and as in every case the microscopic appearance of the

two lobes in the same animal was found to be identical it was decided to use one lobe for the experiment and the other as a control.

Only male rabbits were used and for a short period before the experiment was commenced they were given a diet consisting of raw cabbage which was continued throughout the time the experiment was in progress. In the survival experiments the preparatory technique at each operation was the same. The animal was anesthetised with ether, the neck shaved, and alcohol used as an antiseptic. Iodine was strictly avoided during the operation and we carefully guarded against the possibility of the animal obtaining any iodine supply while under observation. The animals were killed at varying intervals of time. When the time came it was anesthetised with chloretone and exsanguinated. We had previously compared the histology of normal animals so treated with that of similar animals which had been killed by a blow on the occiput, and were unable to detect any alterations due to this anesthetic. In the experiments in which the various nerves were stimulated the animals were similarly anesthetised with chloretone. The nerves were isolated, placed on platinum electrodes, and kept moist throughout the experiment. Instantaneous faradic stimuli were applied to the nerve at the rate of 20 per minute by means of a metronome connected with an induction coil. The criterion of the efficacy of the stimulation in the case of the sympathetic was dilatation of the pupil following stimulation, while stimulation of the vagus gave rise to twitching of the larynx and pharynx on that side. In all the sympathetic experiments and many of the vagal experiments artificial respiration was employed. The tracheotomy tube was inserted low in the neck and damage to the thyroid veins was carefully avoided. The animal was exsanguinated after the experiment was completed. The tissues to be examined were immediately fixed with Müller's fluid to which had been added 5 per cent formalin and the sections were stained with Weigert's iron-hematoxylin and eosin. Occasionally neither lobe was of uniform structure and in these cases we compared average fields from both lobes. Infection was rare in these experiments but when it occurred the result was discarded.

The Normal Thyroid of the Rabbit.

A number of experiments were performed to study the normal histology of the gland in the rabbit under conditions similar to those which prevailed during our experiments. The striking feature of these observations was the marked differences which were seen in different rabbits although in all the animals the histological picture was the same in both lobes. All these observations were made at the same period of the year so that a seasonal variation could not explain the differences. No detailed protocols are given as the histological

descriptions given in the tables of the experiments correspond to the pictures seen in the normal thyroid. The figures shown also illustrate well the type of variation which was found. In some animals the acini were large and filled with colloid which stained well (Fig. 1), while in others the acini were very small and contained much less colloid (Fig. 2). There was often a good deal of irregularity both in the size and shape of the vesicles, and the staining power of the colloid also varied. The cells in the largest acini were usually flat, while in the others they were cuboidal, but cylindrical cells were never seen. Some thyroids showed very marked vacuolation of the colloid, whereas in others this was completely absent. Epithelial cells were often seen in small clumps in the intervesicular tissue. This was present so frequently that it seems to be part of the normal picture in the thyroid of the rabbit. In a few instances small cellular invo\ uns into the lumen of some of the vesicles were seen. Intermediate pictures between these two extremes were seen most frequently (Fig. 3).

The Effect of Drugs.

Before we fully realized the marked individual differences in the thyroid of normal rabbits we endeavored to study the function of the gland by repeated injections of adrenalin, pilocarpine, and strychnine. The pictures which were obtained varied and were within the range of those seen in the normal gland. It was seen that this method of studying the problem could lead to no conclusive results and that the only method by which results could be obtained was to carry out the experiment on one lobe and use the other as a control.

The Effect of Nerve Division.

Three experiments were performed in which the right vagus was divided above the level at which the superior laryngeal nerve is given off, and three in which the right sympathetic cord was divided as low in the neck as possible. In two other experiments the right sympathetic cord, the right recurrent laryngeal nerve, and the depressor nerve were cut, as it was thought that some sympathetic fibers might gain access to the thyroid by way of the recurrent laryngeal. Finally one experiment was carried out in which the right vagus, the

superior laryngeal nerve, and the right sympathetic cord were divided. The animals were killed at intervals varying from 7 to 35 days. Protocols of the experiments and of the histological pictures seen in the two lobes are given in Table I. The results obtained from these experiments were entirely negative, as in every instance the histological appearance of the two lobes was the same.

The Effect of Nerve Stimulation.

In four experiments the right stellate ganglion was exposed posteriorly by Sherrington's rib resection method. The sympathetic cord below the ganglion and the cardiac branches from the ganglion were divided and stimulation was applied to the cord immediately above the ganglion. Three experiments were carried out in which the right sympathetic cord was cut as low in the neck as possible and stimulated at this point. In two instances the right vagus was divided above the superior laryngeal nerve and again below the level at which the recurrent laryngeal nerve is given off. The electrodes were applied to the nerve at the junction of the superior laryngeal and the main trunk. Two further experiments were performed in which the vagus was divided above and below the origin of the superior laryngeal nerve while the recurrent laryngeal nerve was also divided. Both nerves were then stimulated. Stimulation was maintained for periods varying from 1 to 6 hours. No alteration in the microscopic appearance could be observed between the lobe which had been stimulated and the control lobe (Table II).

DISCUSSION.

In the experiments which have been carried out we have been unable to find any histological difference in the thyroid gland either from cutting off the nerve supply or from nerve stimulation. We fully realize that histological study is not the ideal method for investigating the functions of an organ, but we think that this method is more useful in the case of the thyroid gland than with most organs of the body because very slight morphological changes can be detected and, as Marine and his coworkers (41, 42) have shown, there is a close relation between the histological structure and the iodine con-

TABLE I.

Experiment No.	Time killed after operation.	Weight before operation.	Weight when killed.	Left lobe.							Right lobe.
				Vesicles.		Colloid.		Vacuolation.	Cells.	Cellular involution.	
				Size.	Shape.	Amount.	Staining.				
Right vagus divided above superior laryngeal.											
	days	*gm.*	*gm.*								
1	7	2080		Medium and regular.	Regular.	Moderate.	Good.	Marked.	Cuboidal.	Absent.	No difference.
2	28	1960	2025	Irregular.	Irregular.	"	"	Slight.	"	"	" "
3	28	1560	1520	Medium and regular.	Fairly regular.	Fairly large.	"	"	"	"	" "
Right sympathetic cord divided low in neck.											
4	35	1995		Small and some elongated scattered throughout.	Regular; elongated vesicles irregular.	Small.	Faint.	Marked.	Cuboidal.	Absent.	No difference.
5	28	2350	2220	Irregular.	Regular.	Varied greatly.	Rather faint.	Slight.	"	"	Contained rather more colloid.
6	7	2150	2120	Very irregular; some very large.	Very irregular.	Varied considerably.	Varied.	Marked.	"	"	No difference

Right sympathetic cord, right depressor, and right recurrent laryngeal divided.

7	7	1730	1820	Medium; some very large.	Most regular; large irregular.	Large vesicles well filled; others had much less.	Good.	Slight.	Large vesicles flat; others cuboidal.	Absent.	No difference.
8	28	1870	1500	Medium and regular.	Regular.	Moderate.	"	Marked.	Cuboidal.	"	" "

Right vagus, right superior laryngeal, and right sympathetic cord divided.

9	35	2000	1460	Medium and regular with a few larger scattered irregularly.	Regular.	Moderate.	Good.	Moderate.	Cuboidal.	Absent.	No difference.

TABLE II.

Experiment No.	Weight.	Duration of stimulation.	Left lobe.							Right lobe.
			Vesicles.		Colloid.		Vacuolation.	Cells.	Cellular involution.	
			Size.	Shape.	Amount.	Staining.				
			Right stellate ganglion stimulated.							
	gm.	*hrs.*								
10	1950	6	Large and irregular.	Fairly regular.	Large.	Faint.	Absent.	Cuboidal.	Absent.	No difference.
11	1900	1	Medium and regular.	Regular.	Small.	"	"	"	"	" "
12	2000	6	Small and regular.	"	"	Good.	Slight.	"	"	" "
13	2100	1	Mostly small; some large.	"	Large well filled; small contained little.	"	Absent.	"	"	" "
			Right sympathetic cord stimulated low in neck.							
14	1250	5	Medium and regular.	Regular.	Moderate.	Good.	Absent.	Cuboidal.	Absent.	No difference.
15	1600	1	Irregular, some being large—some small.	Fairly regular.	Varied.	"	"	"	"	" "
16	1250	1	Small and regular.	Regular.	Small.	Faint.	"	"	"	" "

Right vagus stimulated above superior laryngeal.										
17	2080	5½	Irregular, some large, some small.	Fairly regular.	Large.	Faint.	Moderate.	Cuboidal.	Absent.	No difference.
18	2300	1	Small and regular.	Regular.	Small.	Fairly good.	Marked.	"	"	" "
Right superior laryngeal and right recurrent laryngeal stimulated.										
19	2360	1	Many large, many small.	Irregular.	Large well filled; small contained less.	Good.	Very slight.	Cuboidal in smaller vesicles; flatter in large.	At some points in both lobes small cell accumulations were seen which projected to a slight extent into the vesicle.	No difference.
20	1950	6	Medium and regular; some large.	Regular; large irregular.	Large well filled; medium contained moderate amount.	"	Absent.	Cuboidal.	Absent.	" "

tent of the gland. Our experiments have clearly demonstrated the marked variations which are present in the microscopic picture of the normal thyroid gland in different rabbits. This difference was seen mostly in the size and shape of the vesicles, the quantity and staining power of the colloid, and the amount of vacuolation. This individual variation in the microscopic appearance of the thyroid gland had been noted in 1888 by Hale White (43) in material he obtained from autopsies on patients who during life had shown no signs of thyroid disease, and other observers have reported similar findings in the course of experimental work. This variable histological picture seems to us to be of great importance in the interpretation of experimental results. The histological evidence which has been brought forward in support of the secretory innervation of the thyroid has been based principally on the structure of the particular lobe which was examined and not on a comparison of the two lobes of the same animal. In view of the marked individual differences which are present normally this evidence cannot be accepted as leading to any definite conclusions. This same criterion applies to experiments in which the gland was studied histologically after drug injection. The results of stimulation of the sympathetic over a period of months reported by Reinhard are quite at variance with his interpretation. He considered that they indicated hyperactivity, whereas the histological picture tended towards that of a colloid rather than a hyperactive gland. Excision of the cervical sympathetic has been frequently performed for the relief of exophthalmos in exophthalmic goiter but with few exceptions no change has been reported either in the exophthalmos or in the thyroid.

The evidence of an altered excitability of the autonomic nervous system following injection of thyroid extracts or stimulation of the cervical sympathetic also cannot be held as proof of an increased outpouring of thyroid secretion after nerve stimulation, as similar experiments by Sharpey-Schafer and Dryerre in which thyroid extracts were injected gave entirely negative results. The experiments of Lieb and Hyman showed a gradual increase in the excitability of autonomic nerves following repeated injection of a constant small dose of adrenalin after thyroidectomy and throw considerable doubt on the experiments in which adrenalin was used as a test of this ex-

citability. The parallelism which Cannon and Smith draw between the action on the denervated heart of massage of the thyroid and sympathetic stimulation does not necessarily prove that the nervous impulses caused an increased outpouring of secretion. Even if one grants that thyroid secretion can alter the rate of the denervated heart it seems possible that the effect of the intermittent stimulation of the thyroid was to produce an alternate contraction and dilatation of the vessels which was in reality a massage. Complete anemia of the gland for the period of the experiment could not be a suitable control for experiments when intermittent stimulation was used. This massaging action might explain also the alteration in the iodine content of a lobe following stimulation of the sympathetic although all authors do not agree that this difference exists. The experiments which have been carried out by Cannon and his coworkers on the production of "action currents" in the thyroid gland as indicating active secretion are interesting but it seems to us that the state of knowledge in regard to the physiological significance of "action currents" is so unsatisfactory that no definite conclusions can be drawn at present from experiments of this nature. The studies which have been made on the changes which take place in thyroid grafts in various parts of the body show conclusively that nerve control is not essential to the adequate functioning of the thyroid gland, therefore any influence which nerves can have is only of an accessory nature. At present one must decide that although the thyroid undoubtedly is supplied by nerves there is no conclusive evidence as to their function.

CONCLUSIONS.

1. There is a marked individual variation in the histological structure of the thyroid gland of rabbits examined at the same season under standard conditions. Although the microscopic appearance varies in different rabbits, the structure of the two lobes in the same animal does not differ. The variations are seen principally in the size and shape of the vesicles and in the quantity and character of the colloid.

2. With one lobe as a control no histological changes in the other lobe have been observed following section or stimulation of either the cervical sympathetic or the vagus and its branches.

BIBLIOGRAPHY.

1. Andersson, O. A., *Arch. Anat. u. Entwcklngsgesch.*, 1894, 177.
2. Berkley, H. J., *Johns Hopkins Hosp. Rep.*, 1895, iv, 281.
3. Rhinehart, D. A., *Am. J. Anat.*, 1912, xiii, 91.
4. Wyss, O., *Cor.-Bl. schweiz. Aerzte*, 1889, xix, 178.
5. Andersson, O. A., *Arch. Anat. u. Entwcklngsgesch.*, 1894, 201.
6. Osokin, N. Y., *Russk. Vrach*, 1915, xiv, 300.
7. Schmid, E., *Arch. mikr. Anat.*, 1896, xlvii, 181.
8. Katzenstein, J., *Arch. Physiol.*, 1897, 371.
9. Missiroli, A., *Arch. fisiol.*, 1909, vi, 588.
10. Lübcke, O., *Virchows Arch. path. Anat.*, 1902, clxvii, 490.
11. Wiener, H., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 310.
12. Reinhard, W., *Virchows Arch. path. Anat.*, 1925, ccliv, 507.
13. Reinhard, W., *Deutsch. Z. Chir.*, 1923, clxxx, 193.
14. Schiff, E., and Heinrick, K. A., *Deutsch. med. Woch.*, 1924, l, 1756.
15. Wilson, L. B., *Am. J. Med. Sc.*, 1918, clvi, 553.
16. Wilson, L. B., and Durante, L., *J. Med. Research*, 1916, xxxiv, 273.
17. Manley, O. T., and Marine, D., *J. Am. Med. Assn.*, 1916, lxvii, 260.
18. Loeb, L., and Hesselberg, C., *J. Med. Research*, 1919, xl, 265.
19. Kummer, E., *Endocrinology*, 1917, i, 222.
20. Asher, L., and Flack, M., *Z. Biol.*, 1910–11, lv, 83.
21. Asher, L., and von Rodt, W. E., *Zentr. Physiol.*, 1912–13, xxvi, 223.
22. Ossokin, N., *Z. Biol.*, 1913–14, lxiii, 458.
23. Oswald, A., *Zentr. Physiol.*, 1915, xxx, 509.
24. Biedl, A., Innere Sekretion, Berlin and Vienna, 2nd edition, 1913, i, 247.
25. Sharpey-Schafer, E., The endocrine organs, London and New York, 2nd edition, 1924, i, 43.
26. Dryerre, A., *Quart. J. Exp. Physiol.*, 1923, suppl., 110.
27. Levy, R. L., *Am. J. Physiol.*, 1916, xli, 492.
28. Lieb, C. C., and Hyman, H. T., *Am. J. Physiol.*, 1922–23, lxiii, 68.
29. Cannon, W. B., and Smith, P. E., *Am. J. Physiol.*, 1922, lx, 476.
30. Cannon, W. B., and Cattell, McK., *Am. J. Physiol.*, 1916, xli, 58.
31. Cannon, W. B., and Cattell, McK., *Am. J. Physiol.*, 1916, xli, 74.
32. Cannon, W. B., Binger, C. A. L., and Fitz, R., *Am. J. Physiol.*, 1914–15, xxxvi, 363.
33. Langley, J. N., and Anderson, H. K., *J. Physiol.*, 1904, xxx, 439.
34. Marine, D., Rogoff, J. M., and Stewart, G. N., *Am. J. Physiol.*, 1917–18, xlv, 268.
35. Troell, A., *Arch. Int. Med.*, 1916, xvii, 382.
36. Burget, G. E., *Am. J. Physiol.*, 1917, xliv, 492.
37. Marine, D., *Arch. Int. Med.*, 1923, xxxii, 811.
38. Rahe, J. M., Rogers, J., Fawcett, G. G., and Beebe, S. P., *Am. J. Physiol.*, 1914, xxxiv, 72.

39. Watts, C. F., *Am. J. Physiol.*, 1915, xxxviii, 356.
40. van Dyke, H. B., *Am. J. Physiol.*, 1921, lvi, 168.
41. Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349.
42. Marine, D., and Lenhart, C. H., *Arch. Int. Med.*, 1909, iii, 66.
43. White, W. Hale, *Med.-Chir. Tr.*, 1888, lxxi, 181.

EXPLANATION OF PLATE 1.

FIG. 1. Experiment 19. Right vagus and right recurrent laryngeal nerves stimulated for 1 hour; 20 stimuli per minute.

FIG. 2. Experiment 4. Right sympathetic cord divided low in neck; killed 5 weeks later.

FIG. 3. Experiment 11. Right stellate ganglion stimulated for 1 hour; 20 stimuli per minute.

Right lobe. Left lobe.

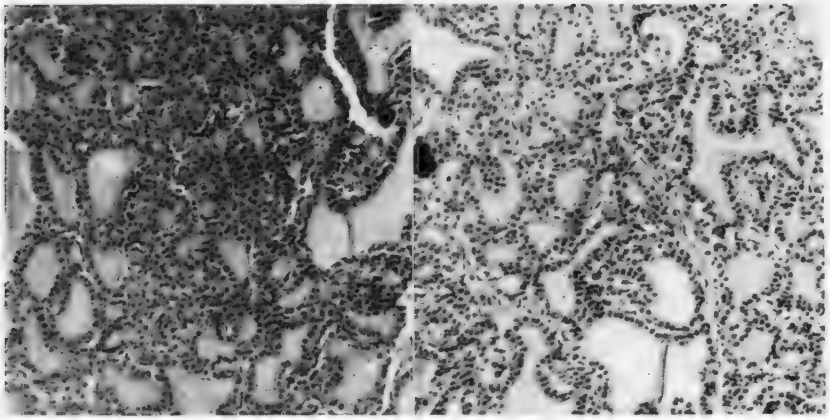


FIG. 1.

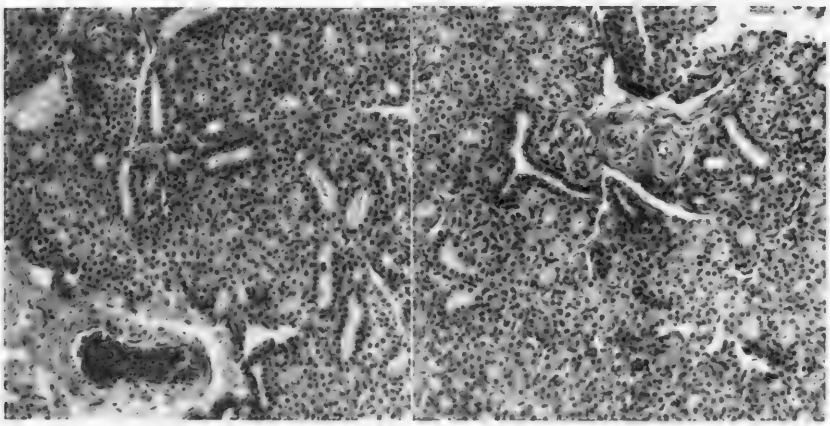


FIG. 2.

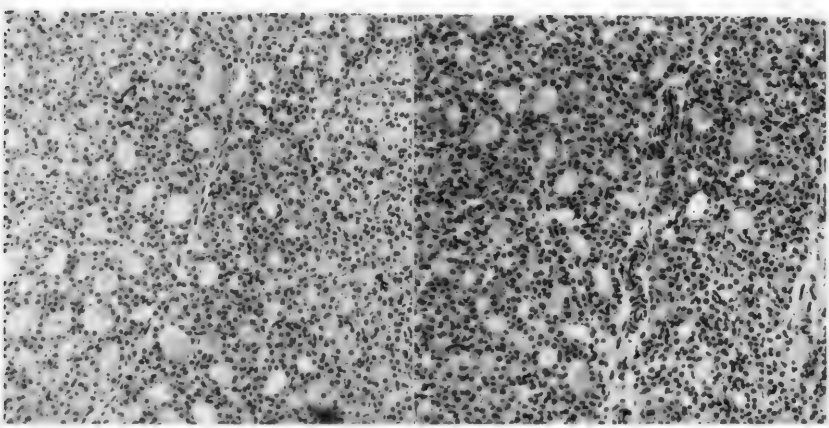


FIG. 3.

(Crawford and Hartley: Autonomic nervous system and thyroid gland.)

THE HISTOLOGICAL CHANGES IN THE THYROID GLAND OF THE RABBIT FOLLOWING LOBECTOMY.*

By J. HAMILTON CRAWFORD, M.D., and J. N. J. HARTLEY, F.R.C.S.

(*From the Pharmacology Department of Edinburgh University, Edinburgh, Scotland.*)

Plate 2.

(Received for publication, May 13, 1925.)

Since the observations reported by Reverdin and Reverdin (1) and Kocher (2) that total thyroidectomy produced symptoms of operative myxedema or cachexia strumipriva many observers have studied the effect of total and partial thyroidectomy in animals. Wagner (3) performed lobectomy in a dog and in a cat and stated that the remaining lobe increased in size. Fuhr (4), who removed the remaining lobe from 8 to 41 days after the first operation, was unable to confirm this, but Horsley (5) found that after removal of one lobe the other may be four or five times its normal size 160 days later. The cells in the hypertrophied lobe were increased in size and there was plication of the acinus wall while the colloid became less solid. Von Eiselsberg (6) removed one lobe and 3 to 15 days later removed the other, but says he found no naked eye appearance of hypertrophy. In two cases in which the remaining lobe was not removed till 21 and 115 days after the first lobe had been excised he states that he got no evidence of hypertrophy. Halsted (7) carried out a very extensive series of investigations on hypertrophy of the thyroid following partial thyroidectomy. Most of these experiments were of a piecemeal nature, the amount of thyroid tissue being gradually reduced at successive operations. He, however, carried out a number of experiments in which one lobe was excised and the other examined at periods varying from 3 hours to 132 days. No change was observed as a rule before the 22nd day, after which all the glands examined were abnormal. Previous to this in only four experiments was there evidence of early hyperplasia. In one of these the hyperplasia was seen after 7 days, in another after 15 days, and in two others after 19 days. The histological changes which indicated hypertrophy were, increase in size of the epithelium, cellular involution into the vesicles, and thin vacuolated colloid. The changes which were observed in these experiments seemed to bear no relation to the quantity of tissue removed. In a later communication Halsted (8) states that he repeated these experiments under strictly antiseptic precautions in two dogs and got negative results. Under his direction Hunnicutt (9)

* The expenses of this research were defrayed by a grant from the Earl of Moray Fund, Edinburgh University.

carried out a further series of experiments and found no evidence of hypertrophy. In most, although not all, of these experiments iodine had, however, been used as a skin antiseptic. Marine and his coworkers (10) confirmed Halsted's original work and found that there was a definite relation between iodine content and hypertrophy. They also showed that iodine administration could prevent hypertrophy taking place and also cause reversion of a hypertrophied gland to one of a colloid type. In view of Hunnicutt's findings Marine repeated his earlier work but obtained the same results as before. He thinks that the negative results were due to traces of iodine (11). Loeb (12) performed a series of experiments on guinea pigs in which he excised one lobe and studied the histological changes in the other at periods varying from 4½ days to 4 months. He found no evidence of definite hypertrophy, but changes were present, especially in the later stages which he thought represented the early stages of hypertrophy.

The present investigation was undertaken with a view to studying the influence which the innervation of the thyroid played in the changes which took place in the gland following lobectomy. The effects of hemithyroidectomy on the remaining lobe were first studied. Experiments were then performed in which the same procedure was adopted and in addition the different nerves to the remaining lobe were divided. The right lobe was excised in every instance. In all these experiments only male rabbits were used and the rabbits were kept on a diet of raw cabbage for a short time before the experiment and throughout the period of observation. The operative technique was similar to that described in a companion paper, particular care being taken to avoid the animals obtaining a supply of iodine either at the operation or during the experiment. The operations were performed under ether anesthesia. All the wounds healed without suppuration. The animals were killed at varying intervals of time following the operation and the tissues were fixed and stained as described in our other paper.

Although operative interference in the region of the neck had been demonstrated in previous experiments not to give rise to changes in the thyroid gland it was thought wise to perform a control experiment in which only a small portion of the thyroid gland was removed, in order to see whether interference with the thyroid itself altered the histological picture. The animal was killed 7 days later but no histological difference could be detected between the two lobes, in marked contrast to the changes observed at the end of the same period in the experiments described below.

The Effect of Excision of the Right Lobe on the Histology of the Left Lobe.

This series consisted of five experiments and the animals were killed at periods varying from 2 days to 3 months after the operation. The structure of the lobe removed by operation was compared with that of the lobe removed at the completion of the experiment, and the findings are described in Table I. The microscopic appearance of the normal lobe varied in different animals in a manner similar to that described in our other paper. These experiments showed that lobectomy was followed by a series of histological changes in the remaining lobes. 2 days after the removal of the right lobe the vesicles of the left lobe were slightly increased in size, its colloid more vacuolated, and the epithelial cells slightly larger. After 4 days the vesicles were increased in size and contained more colloid which stained moderately well and was not vacuolated (Fig. 1). At the end of 7 days the vesicles were distended with deeply staining colloid and the glandular cells tended to be flattened (Fig. 2). After the lapse of 3 months the histological picture of the left lobe resembled that of the lobe previously removed. The vesicles were no longer distended with colloid but in some cases contained less than those of the right lobe while the glandular cells were cuboidal and somewhat larger (Fig. 3). The remaining lobe had increased in weight and this was presumably due to an increase in the number of vesicles.

The Effect of Nerve Division on the Histological Changes after Lobectomy.

This series consisted of five experiments in which a right hemithyroidectomy was performed and at the same time various nerves on the left side divided. In three experiments the left vagus was divided above the origin of the superior laryngeal nerve, while in the remaining two, the left sympathetic cord was cut as low in the neck as possible. The rabbits were killed at periods varying from 7 days to 4 months and the histological changes which took place are shown in Table II which gives the protocols of the experiments. The histological appearance, both in the experiments in which the vagus was cut and in those in which the sympathetic had been divided, was similar in every respect to that seen at a corresponding time when the nerves were intact.

TABLE

Experiment No.	Time killed after lobectomy.	Weight before lobectomy.	Weight when killed.	Vesicles.			
				Size.		Shape.	
				Right.	Left.	Right.	Left.
	days	*gm.*	*gm.*				
21	2	1450	1485	Small and regular.	Slightly larger.	Regular.	Same.
22	4	1495	1495	" "	Many large.	"	Slightly irregular.
23	7	2100	1930	Medium and regular.	Much larger.	"	Same.
24	90	1620	1830	Irregular; many large.	Same.	Large, irregular.	"
25	45	1400	1240	Medium and regular.	"	Regular.	"

TABLE

Experiment No.	Time killed after operation.	Weight before operation.	Weight when killed.	Vesicles.			
				Size.		Shape.	
				Right.	Left.	Right.	Left.
							Left vagus divided
	days	*gm.*	*gm.*				
27	7	1720	1180	Irregular; some large.	Much larger.	Slightly irregular.	More regular.
28	30	1970	1750	Irregular; most very large.	Much smaller.	Regular.	Slightly irregular.
29	120	1600	2000	Medium.	Same.	"	Same.
							Left sympathetic cord
30	7	2050	1855	Small.	Very large.	Regular.	Irregular.
31	120	1920	2100	Small a few medium.	Same.	"	Same.

I.

Colloid.				Vacuolation.		Cells.		Cellular involution.	
Amount.		Staining.							
ight.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.
nty.	Slightly increased.	Faint.	Same.	Slight.	More marked.	Cuboidal.	Slightly larger.	Absent.	Absent.
all.	Increased.	"	"	"	Absent.	"	Same.	"	"
der- e.	Much increased.	Good.	Darker.	Moderate.	Increased.	"	Flat.	"	"
"	Less.	"	Slightly fainter.	Absent.	Slight.	Cells flat in large vesicles; others cuboidal.	Columnar.	At a few points there was slight cell involution into vesicles.	Same.
"	Same.	"	Same.	Slight.	Same.	Cuboidal.	Slightly larger.	Absent.	Absent.

II.

Colloid.				Vacuolation.		Cells.		Cellular involution.		Remarks.
Amount.		Staining.								
Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	Right.	Left.	
above superior laryngeal.										
irly arge.	Increased.	Good.	Same.	Marked.	Same.	Cuboidal.	Flat.	Absent.	Absent.	Marked inspiratory stridor.
rge.	Less.	"	"	A few large vacuoles.	Absent.	Flat.	Cuboidal.	"	"	
oder- te.	Slightly less.	Fairly good.	"	Marked.	Less marked.	Cuboidal.	Larger.	"	"	
divided low in neck.										
all.	Large.	Faint.	Same.	Slight.	Slightly increased.	Cuboidal.	Flat.	Absent.	Absent.	Left lobe showed cellular projections into some vesicles.
oder- te.	Same.	Good.	"	Moderate.	Same.	"	Same.	"	"	

DISCUSSION.

The fact that signs of hyperplasia are present in the remaining lobe 3 months after one lobe has been removed has been frequently observed, but the change which we observed during the first 2 weeks has not been reported by other authors. Few observations have been made, however, at an early date after lobectomy, as most investigators have been interested in compensatory hypertrophy of the gland and not in the early changes. Therefore they did not remove the second lobe until a long time after lobectomy had been performed. The changes which took place at this time are not easy to explain. We do not think that they were due to the animals having obtained a supply of iodine, as we carefully avoided this possibility both during and after the operation. They also do not appear to be a reaction to operation on the neck since such changes were not present in a series of experiments which we performed in which the nerves only were divided, nor in the control experiment in which only a portion of one lobe was removed. The fact that 2 days after the operation the remaining lobe showed a slight increase in size of the vesicles with more marked vacuolation of the colloid and a slight increase in size of the cells lining the vesicles probably indicated an increase in activity. The appearance of the gland at the end of a week was similar to that of a colloid gland, which Marine considers to be the resting state of the thyroid following hyperactivity and the nearest approach to normal which can take place in a gland which has been hyperactive. No studies of basal metabolism were made which would indicate if there had been a primary state of hyperactivity followed by a resting state. The iodine content was not estimated so that we cannot say definitely that the glands at the end of 7 days were of a true colloid nature. It would, therefore, be impossible to venture a definite explanation. We consider it possible, however, that the sudden demand on the thyroid resulting from hemithyroidectomy has caused the thyroid cells to hyperfunction. At the end of 7 days, the demand having been satisfied, it has assumed a resting stage. Thereafter compensatory hypertrophy has gradually become established by an increase in the number of vesicles with a resumption of the normal histological picture.

The changes which occurred were uninfluenced by division of the nerves which pass to the remaining lobe so that the experiments lend no support to the view that the function of the thyroid is controlled by nervous influences.

CONCLUSIONS.

1. In a series of rabbits the changes which took place in the histology of the left lobe of the thyroid gland after excision of the right lobe have been studied at varied intervals of time. 2 days after the removal of one lobe the other lobe showed a slightly increased colloid content, an increased vacuolation of the colloid, and a slight increase in the size of the epithelial cells. At the end of a week the vesicles were greatly increased in size, distended with colloid, and the epithelial cells were flattened, while after 3 to 4 months signs of compensatory hypertrophy were present.

2. The changes observed were uninfluenced by division of the vagus or cervical sympathetic.

BIBLIOGRAPHY.

1. Reverdin, J. L., and Reverdin, A., *Rev. méd. Suisse romande*, 1883, iii, 169, 233, 309, 413.
2. Kocher, T., *Arch. klin. Chir.*, 1883, xxix, 254.
3. Wagner, J., *Wien. med. Bl.*, 1884, vii, 932.
4. Fuhr, F., *Arch. exp. Path. u. Pharmakol.*, 1886, xxi, 387.
5. Horsley, V., *Lancet*, 1886, ii, 1163; *Brit. Med. J.*, 1892, i, 215, 265, 1113.
6. von Eiselsberg, A., Über Tetanie in Ausschlusse an Kropfoperationen, Vienna, 1890.
7. Halsted, W. S., *Johns Hopkins Hosp. Rep.*, 1896, i, 373.
8. Halsted, W. S., *Tr. Assn. Am. Phys.*, 1913, xxviii, 127.
9. Hunnicutt, J. A., *Am. J. Med. Sc.*, 1914, cxlviii, 207.
10. Marine, D., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 359. Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 349. Marine, D., and Lenhart, C. H., *Bull. Johns Hopkins Hosp.*, 1909, xx, 131.
11. Manley, O. T., and Marine, D., *Proc. Soc. Exp Biol. and Med.*, 1914–15, xii, 202.
12. Loeb, L., *J. Med. Research*, 1919, xl, 199.

EXPLANATION OF PLATE 2.

FIG. 1. Experiment 22. Right lobe excised; left lobe 4 days later.
FIG. 2. Experiment 23. Right lobe excised; left lobe 7 days later.
FIG. 3. Experiment 24. Right lobe excised; left lobe 90 days later.

Right lobe. Left lobe.

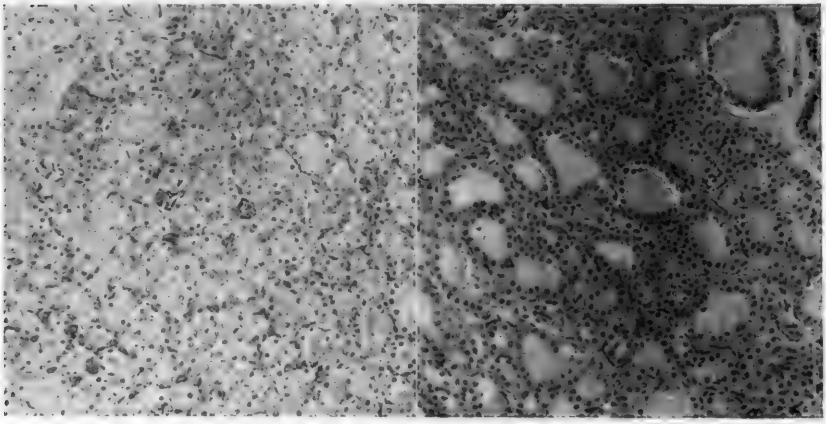


FIG. 1.

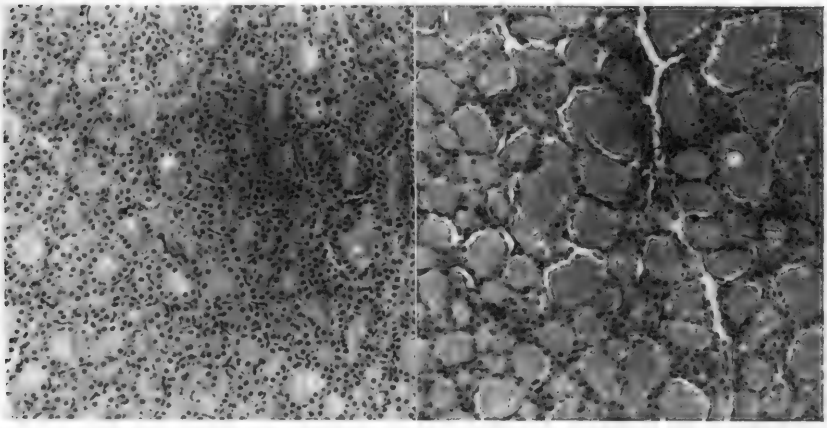


FIG. 2.

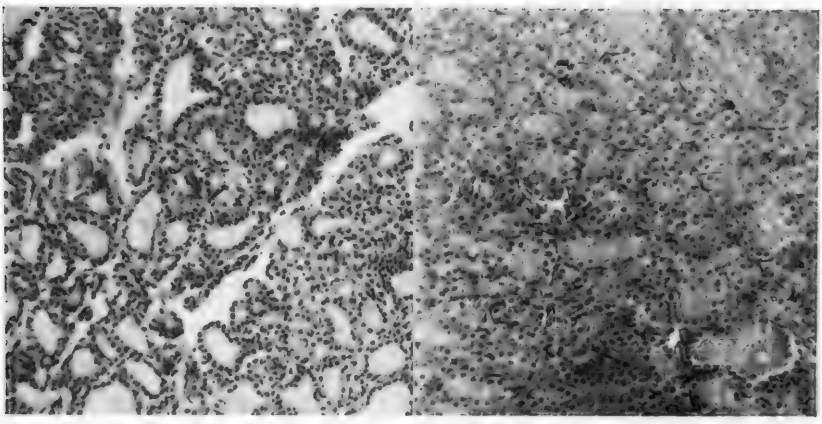


FIG. 3.

(Crawford and Hartley: Thyroid gland after lobectomy.)

THE FUNCTION OF MACROPHAGES IN LOCAL RESISTANCE TO BACTERIAL INFECTIONS.

By WARO NAKAHARA, D.M.Sc.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 3.

(Received for publication, May 14, 1925.)

That intraperitoneal or intrapleural injections of fatty oil bring about a marked mononuclear exudation is a well established fact.[1,2] Although the large mononuclear cells which predominate in the cellular population of the exudate were once considered as of the lymphoid series, a closer study by means of supravital technique has demonstrated them to be macrophages (clasmatocytes and monocytes).[3]

Metschnikoff[4] early distinguished the large mononuclear phagocyte under the name of macrophage from the polymorphonuclear leucocyte (microphage) on the ground that it does not phagocytize bacteria, except for the tubercle bacillus and the bacillus of leprosy, and engulfs only cellular débris. This conception has long since been found to be erroneous, and the phagocytic action of macrophages upon bacteria in general, in addition to that upon pigments, foreign particles, etc., is now a generally accepted fact. Furthermore, there are some indications pointing to the active rôle of macrophages in defensive processes, not only against chronic infections, but also against acute phenomena as well.[5,6] Gay and Morrison[7] only recently showed that in a certain streptococcus infection macrophages (clasmatocytes), with the exclusion of polymorphonuclear cells, are active agents

[1] Bergel, S., *Berl. klin. Woch.*, 1919, lvi, 915; *Ergebn. inn. Med. u. Kinderheilk.*, 1921, xx, 36.

[2] Nakahara, W., *J. Exp. Med.*, 1922, xxxv, 493.

[3] See, for a recent account of this subject: Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 127.

[4] Metschnikoff, E., *Virchows Arch. path. Anat.*, 1887, cvii, 209.

[5] Wallgren, A., *Beitr. path. Anat. u. allg. Path.*, 1899, xxv, 206. Zangemeister, W., and Gans, H., *Münch. med. Woch.*, 1909, lvi, 793.

[6] Buxton, B. H., and Torrey, J. C., *J. Med. Research*, 1906, xv, 55, 73.

[7] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.

in resistance. They noted that the reaction of effective immunity was characterized by a predominance of macrophages in the exudate, while a polymorphonuclear reaction was invariably an accompaniment of a fatal infection. Evidence was presented also that animals in which a rich polymorphonuclear exudate was produced by means of aleuronat, diatomaceous earth, etc., were actually less resistant than normal animals, whereas even a relatively slight macrophage exudate, such as can be produced by plain infusion broth, tended to protect the animal against many multiples of the fatal dose of streptococcus.

The method of inducing a marked macrophage reaction in the peritoneal cavity by means of oil injection offers an excellent opportunity for the study of the function of these cells, and advantage was taken of this method in experiments to be presented in this paper. With the hope of elucidating somewhat the significance of the macrophage reaction in resistance to bacterial infections, I have first attempted to determine the influence of the cellular reaction on the survival of bacteria and then to ascertain to what extent the local resistance thus induced would affect the course of the eventual general infection.

Production of Macrophage Reaction by Oil Injection.

For the purpose of inducing the cellular reaction commercial olive oil was used in all the experiments. This substance was injected intraperitoneally into mice in a single dose of 0.2 cc.

Following the oil injection, there is a considerable polymorphonuclear reaction. By the 48 hour period, however, the leucocytes are largely replaced by mononuclear elements. These latter, consisting chiefly of macrophages, are found in very large numbers during the next few days and this reaction gradually subsides in the course of 2 weeks. Some idea of the macrophage reaction may be obtained from the few examples cited below (Table I). The percentage changes are based on differential cell counts on smears obtained by the capillary method before and at intervals after oil injections. It should be mentioned that oil injections bring about no perceptible increase in the total cell counts. In our experience these latter varied between 100,000 to 180,000 cells per c. mm. of peritoneal fluid in normal and oil-injected mice alike. The total amount of fluid appeared to be slightly increased in the treated animals, however.

TABLE I.

Intraperitoneal Macrophage Reaction Induced by Oil Injection.

Mouse No.	1 day before oil injection.	48 hrs. after oil injection.	5 days after oil injection.
	per cent	*per cent*	*per cent*
1	2.8	72.7	76.6
2	6.2	76.9	80.1
3	14.8	64.8	73.6
4	21.0	87.4	86.5
5	21.7	68.3	78.8
Average.....	13.3	74.0	79.1

We have previously noted that accompanying this local reaction there is a proliferation of the cells of the lymphoid germ centers, but that no material change in the blood picture occurs.[2]

Effect of Oil Injection on the Survival of Bacillus coli in the Peritoneal Cavity.

The first experiments were planned to test the effect on bacteria of the macrophage reaction induced by oil injection. In these experiments, as in others to be described later, olive oil was injected 3 or 4 days in advance of bacterial inoculation, this period being that of the maximum cellular reaction. For obvious reasons a relatively non-pathogenic bacterium was chosen as the test organism; namely, *Bacillus coli.*

Experiment 1.—60 normal white mice of approximately the same size and weight were divided into 4 groups:

Group 1 (20 mice) was injected intraperitoneally with olive oil, and 3 days later was inoculated, also intraperitoneally, with a suspension of *B. coli.*

Group 2 (20 mice) was inoculated intraperitoneally with the same suspension of *B. coli*, without the previous oil injection.

Group 3 (10 mice) was injected with olive oil but was not inoculated with *B. coli.*

Group 4 (10 mice) remained without oil injection or *B. coli* inoculation as normal controls.

24 hours growth of *B. coli* on 4 agar slants was suspended in 8 cc. of sterile normal salt solution, and 0.1 cc. of the suspension was inoculated into each mouse.

2 days after the bacterial inoculation, 10 mice each from Groups 1 and 2 and all the mice of Groups 3 and 4 were killed and cultures were taken from the peritoneal

exudate. 5 mice each from Groups 1 and 2 were killed 4 days after the inoculation and cultures were taken similarly. The remaining 5 mice each from these two groups were killed and cultures taken at the 6 day period.

After 24 hours incubation at 37°C., tubes were examined and growth of *B. coli* noted. The result was as follows (Table II):

TABLE II.

Effect of Oil Injection on the Recovery of B. coli Subsequently Inoculated.

Intervals after *B. coli* inoculation.	Group 1. Inoculated with *B. coli* 3 days after oil injection.	Group 2. Inoculated with *B. coli* alone.	Group 3. Injected with oil alone.	Group 4. Untreated controls.
days				
	+++	+++	−	−
	++	+++	−	−
	++	+++	−	−
	++	+++	−	−
2	++	++	−	−
	+	++	−	−
	+	++	−	−
	−	++	−	−
	−	++	−	−
	−	++	−	−
	+	++		
	−	++		
4	−	+		
	−	+		
	−	−		
	−	++		
	−	−		
6	−	−		
	−	−		
	−	−		

+++ indicates profuse growth; ++, several colonies; +, colonies present.

As is apparent from the above result, *Bacillus coli* dies off rapidly in the normal peritoneal cavity, but it disappears much more quickly from the cavity previously injected with oil. It may be noted that by the 4th day after inoculation cultures from the oil-injected mice were mostly negative, while those from the control animals showed, in the majority of instances, some growth of the organism. Even

2 days after there was a marked difference in the number of recovered organisms.

Experiment 2.—The above experiment was repeated with 40 mice, similarly divided into 4 groups. In this case, however, all the mice were killed and cultures taken 3 days after the *B. coli* inoculation. In Groups 1 and 2, cultures were also taken from the heart's blood. The dose of *B. coli* was the same as in Experiment 1. The growth of *B. coli* in the cultures after 24 hours incubation is shown in Table III.

TABLE III.

Effect of Oil Injection on the Recovery of B. coli Subsequently Inoculated.

Group 1. Inoculated with *B. coli* 3 days after oil injection.		Group 2. Inoculated with *B. coli*.		Group 3. Injected with oil.	Group 4. Normal controls.
Exudate.	Heart's blood.	Exudate.	Heart's blood.	Exudate.	Peritoneal fluid.
++	−	+++	++	−	−
+	−	+++	++	−	−
+	−	+++	+	−	−
+	−	+++	−	−	−
−	−	++	−	−	−
−	−	++	−	−	−
−	−	++	−	−	−
−	−	+	−	−	−
−	−	+	−	−	−
−	−	−	−	−	−

Does Oil Injection Affect the General Resistance?

The next experiment was carried out with the idea of determining whether the resistance induced by oil injection is confined to the peritoneal cavity or whether it involves a general change. The method of experiment adopted was to inject olive oil intraperitoneally as usual, and then to introduce *Bacillus coli* into the pleural instead of peritoneal cavity.

Experiment 3.—10 normal white mice (Group 1) were injected intraperitoneally with 0.2 cc. of olive oil. 3 days later these, together with another 10 mice (Group 2), were inoculated with an equal amount of a suspension of *B. coli* intrapleurally. All the mice, along with 10 untreated normal controls (Group 3), were killed and cultures were taken from the pleural fluid. Examination of the cultures after 24 hours incubation showed the following result (Table IV).

TABLE IV.

Effect of an Intraperitoneal Oil Injection on the Recovery of B. coli Inoculated Intrapleurally.

Group 1. Inoculated with *B. coli* after oil injection.	Group 2. Inoculated with *B. coli* alone.	Group 3. Normal controls.
+++	+++	−
+++	+++	−
+++	+++	−
+++	+++	−
+++	+++	−
+++	+++	−
+++	++	−
++	++	−
++	++	−
−	+	−

This experiment was repeated with the result entirely in agreement with the above.

The observations seem sufficient for the conclusion that there is no perceptible increase in the general resistance of animals under the conditions of these experiments.

Effect of Local Resistance on the Generalization of Infection.

So far, we have confined ourselves to the study of the survival of relatively non-pathogenic bacteria in the peritoneal cavity as affected by previous oil injections. There is another way in which the question of the local resistance can be approached, and that is to test the effect of the treatment on the dissemination of highly virulent microorganisms from the locus of inoculation. The local resistance at the site of primary infection, if of sufficient degree, should modify the course of the eventual general infection.

The following experiments were performed with the above problem in mind, with *Staphylococcus aureus* and pneumococcus as test organisms.

Staphylococcus. Experiment 4.—40 normal white mice were divided into 2 groups:

Group 1, composed of 20 mice, was injected intraperitoneally with 0.2 cc. of

olive oil. 3 days later they were inoculated, also intraperitoneally, with 0.1 to 0.8 cc. of a suspension of *Staphylococcus aureus*.

Group 2 was not injected with olive oil but was inoculated with corresponding amounts of the same bacterial suspension.

The suspension was made by taking up 24 hours growth of the organism on 4 agar slants in 20 cc. of sterile salt solution.

The death rate and the proportion of surviving mice for different doses of staphylococci inoculated, as contrasted between the oil-injected and non-oil-injected groups, is summarized in Table V. These results were confirmed by another experiment of similar nature. Animals living 7 days after inoculation were considered to have survived the infection in these experiments.

TABLE V.

Effect of Oil Injection on the Resistance of Mice to Staphylococcus aureus.

Amounts of suspension of staphylococci inoculated.	Oil-injected mice.	Control mice.
cc.		
0.8	D. 24 hrs. " 48 " " 3 days. " 3 " S.	D. 24 hrs. " 24 " " 24 " " 24 " " 3 days.
0.4	D. 48 hrs. " 3 days. S. " "	D. 24 hrs. " 24 " " 3 days. S. "
0.2	D. 3 days. S. " " "	D. 48 hrs. " 48 " " 48 " " 3 days. S.
0.1	S. " " " "	D. 3 days. " 4 " " 5 " S. "

D., died; S., survived.

Pneumococcus.—Experiments similar to the above were carried out with a Type I pneumococcus of the Neufeld strain.[8]

The following protocol represents one of two experiments which I have performed, and which have given comparable results.

Experiment 5.—50 normal white mice were divided into 2 groups of 25 each. Group 1 was injected intraperitoneally with 0.2 cc. of olive oil, Group 2 remaining untreated. 3 days later, both groups were inoculated intraperitoneally with 0.000,1 to 0.000,000,01 cc. of an 18 hour broth culture of pneumococci. The outcome of this experiment was as follows (Table VI):

TABLE VI.

Effect of Oil Injection on the Resistance of Mice to Pneumococcus.

Amounts of pneumococcus culture injected.	Oil-injected mice.	Control mice.
cc.		
0.000,1	D. 48 hrs. " 48 " " 48 " " 3 days. " 4 "	D. 24 hrs. " 24 " " 24 " " 48 " " 48 "
0.000,01	D. 48 hrs. " 48 " " 3 days. " 3 " " 4 "	D. 24 hrs. " 48 " " 48 " " 48 " " 48 "
0.000,001	D. 48 hrs. " 3 days. " 3 " " 3 " " 4 "	D. 48 hrs. " 48 " " 48 " " 48 " " 48 "
0.000,000,1	D. 3 days. " 4 " S. " "	D. 48 hrs. " 48 " " 48 " " 48 " " 3 days.
0.000,000,01	S. " " " "	D. 48 hrs. " 48 " " 48 " " 3 days. S.

[8] I am indebted to Dr. O. T. Avery for the cultures of this strain.

Failure of Olive Oil by Itself to Influence Infections.

A question naturally arises: Is not the apparent resistance observed attributable to the action of olive oil itself upon the organisms? We have attempted to answer this question by studying the effect of nearly simultaneous injections of oil and bacteria. If the amount of olive oil injected was sufficient by itself to influence the bacterial growth *in vivo*, this effect should be more strikingly brought out in these experiments than when the oil injection was given a few days in advance of bacterial inoculation, as we have heretofore done. It is also to be remembered that there is no marked macrophage reaction in the peritoneal cavity during the first 24 hours after oil injection.

Effect on the Survival of B. coli. Experiment 6.—20 normal white mice were inoculated intraperitoneally with equal amounts of a suspension of *B. coli*. Immediately afterward 10 of these mice were injected, also intraperitoneally, with 0.2 cc. of olive oil, the remaining 10 mice, not injected with olive oil, serving as controls. 3 days later all the mice were killed and cultures were taken from the peritoneal exudate. The growth of *B. coli* in these cultures after 24 hours incubation was as follows (Table VII):

TABLE VII.

Effect of an Oil Injection on the Recovery of B. coli.

Inoculated with *B. coli* immediately before oil injection.	Inoculated with *B. coli* alone.	Inoculated with *B. coli* immediately before oil injection.	Inoculated with *B. coli* alone.
+++	+++	+	+
+++	+++	+	+
+++	+++	+	+
+++	+++	−	−
++	++	−	−

Another experiment of similar nature was carried out with the same number of mice, and with entirely analogous results.

It is evident from the above results that the possible direct action of olive oil on *Bacillus coli* cannot account for the early disappearance of the organism from the oil-prepared peritoneal cavity as noted in the preceding section.

Effect on Pneumococcus Infection.—As a further check, an additional experiment was performed with pneumococcus, as follows:

Experiment 7.—30 normal white mice were divided into 3 groups of 10 mice each. Group 1 was injected with 0.2 cc. of olive oil and 4 days later was inoculated with 0.000,001 cc. of 24 hours growth of Type I pneumococci in broth. Group 2 was inoculated with the same amount of the same culture but was injected immediately before with 0.2 cc. of olive oil. Group 3 was simply inoculated with the same amount of the same culture. Oil as well as pneumococci was inoculated intraperitoneally in all instances.

The proportions of mice in each group surviving the pneumococcus infection were as follows (Table VIII):

TABLE VIII.

Effect of Oil Injection on the Resistance of Mice to Pneumococcus.

Group 1. Injected with oil 4 days before pneumococcus inoculation.	Group 2. Injected with oil immediately before pneumococci.	Group 3. Untreated mice inoculated with pneumococci.
S.	D. 48 hrs.	D. 48 hrs.
"	" 48 "	" 48 "
"	" 48 "	" 48 "
"	" 48 "	" 48 "
"	" 3 days.	" 48 "
"	" 3 "	" 3 days.
"	" 3 "	" 3 "
"	S.	" 3 "
"	"	S.
"	"	"

Is the Exudation Fluid Bacteriolytic?

At this point it is necessary to consider the possible rôle of the peritoneal fluid itself in the process of local resistance. It is not without reason to suspect that the soaps of fatty acids which may be formed in the peritoneal cavity after oil injection might be of some consequence. Of the three species of bacteria used in the preceding experiments pneumococcus is the only one known to be highly susceptible to the lytic action of sodium oleate, and, even in this case, the soap action is inhibited by the presence of serum.[9] Staphylococcus, on the other hand, is highly resistant to the germicidal action of soaps.[10] In spite of these facts it seems desirable to obtain direct experimental evidence. I have therefore tested peritoneal fluid from

[9] Lamar, R. V., *J. Exp. Med.*, 1911, xiii, 380.

[10] Walker, J. E., *J. Infect. Dis.*, 1924, xxxv, 557.

oil-injected animals for its action on pneumococcus, using rabbits, instead of mice, for technical reasons.

The following is one of two experiments which resulted in an identical outcome.

Experiment 8.—A normal rabbit was injected intraperitoneally with 20 cc. of olive oil. 4 days later the peritoneal exudate was collected and centrifuged, separating oil droplets and cells from the clear fluid. 0.5 cc. of this last mentioned fraction was mixed in a small test-tube with 0.05 cc. of 10 hour broth culture of a passage strain of Type I pneumococcus and placed in a water bath at 37°C.

Microscopical examinations were made of smears from the mixture taken at the end of 1, 3, and 6 hours of incubation. Before incubation smears showed, roughly, 1 organism in 5 to 10 microscopic fields. After 1 hour's incubation 1 or 2 organisms were seen in every field. A marked increase of pneumococci was noted after 3 hours incubation, at which period there were 10 to 20 organisms per field. The bacteria further increased during the next 3 hours of incubation, and as many as 50 of these were seen in a single microscopic field. The original undiluted culture showed as an average 4 or 5 pneumococci per field.

Cultures were also taken from the mixture after incubation for 1, 3, and 6 hours. All the cultures yielded luxuriant growths of pneumococci.

Finally, the mixture, after 6 hours incubation, was inoculated into mice in varying doses. 0.001 cc. of this material uniformly killed mice within 24 hours, and 0.000,01 cc. killed within 48 hours.

The evidence seems complete in demonstrating that the cell-free exudate from an oil-injected animal, instead of inhibiting the growth of pneumococcus, serves, on the contrary, as an excellent culture medium for this organism. This fact makes it certain that the properties of the fluid, including its soap contents, are not primarily responsible for the phenomena of local resistance.

Phagocytosis of Bacteria by Macrophages.

After eliminating the humoral factor from consideration there remains to be described the microscopical evidence of the action of macrophages upon bacteria.

A number of mice were injected with olive oil as in previous experiments and 3 or 4 days later they were inoculated with microorganisms. In inoculating virulent bacteria care was taken to use the amount of culture sufficient to kill normal animals and yet permit the oil-injected ones to survive. With *B. coli*, a small quantity of fairly thick suspension was used. All the animals were killed from

2 to 4 hours after the inoculation of bacteria, and smears were taken from the peritoneal exudate for microscopical examination.

Figs. 1 to 6 illustrate the findings. There were some variations in the relative number of bacteria found in the smear, depending, no doubt, upon the amount of cultures originally injected, upon varying time after the inoculation, etc., but the evidence of phagocytosis of bacteria by macrophages was a very frequent and constant observation. In Figs. 1 and 2 are shown a number of *Bacilli coli* in, as well as about, macrophages; a fair number of *Staphylococcus aureus* are shown phagocytized in Figs. 3 and 4, while several pneumococci engulfed by macrophages are illustrated in Figs. 5 and 6. It was noted that the cells phagocytizing all these microorganisms were chiefly macrophages, and only occasionally were polymorphonuclear leucocytes seen containing any bacteria.

DISCUSSION.

It is well known that virulent microorganisms injected into the peritoneal cavity are rapidly disseminated into the circulating blood, and that the organisms, if allowed to proliferate, lead to the eventual death of animals. At the same time, there is no doubt that many bacteria are disposed of in the peritoneal cavity. The ultimate recovery of the animal may therefore depend to a certain extent upon how far the local destruction of bacteria is carried out. Despite the fact that polymorphonuclear cells are generally accepted as the major agent in these local processes, there are a few observers who consider these cells to be of little or no importance in disposing of bacteria in the peritoneal cavity (Buxton and Torrey,[6] Simon,[11] etc.). On the other hand, it has been suggested that macrophages might be active agents under these conditions (Gay and Morrison[7]).

It is not our intention to discuss here how important a part the macrophage plays in resistance to infections as they occur spontaneously in man and animals. Nor is it within the scope of this paper to question the function of polymorphonuclear cells. We have merely demonstrated in the present investigation the ability of macrophages to successfully dispose of pathogenic bacteria with but slight, if any, cooperation of polymorphonuclear elements.

[11] Simon, F. B., *Centr. Bakt., 1. Abt.*, 1901, xxix, 113.

SUMMARY.

Coincident with the marked macrophage reaction induced in the peritoneal cavity by oil injection, there is an increased resistance of this location to bacterial infections. Animals so prepared dispose of *Bacillus coli* in a much shorter time than normal animals, and survive multiples of the fatal doses of staphylococci and pneumococci.

The amount of oil injected is in itself incapable of inhibiting bacterial growth, nor is cell-free exudate from an oil-injected animal potent in this regard. The macrophages in the exudate, on the other hand, actively phagocytize bacteria. These facts lead to the conclusion that the increased resistance is due primarily to the action of macrophages.

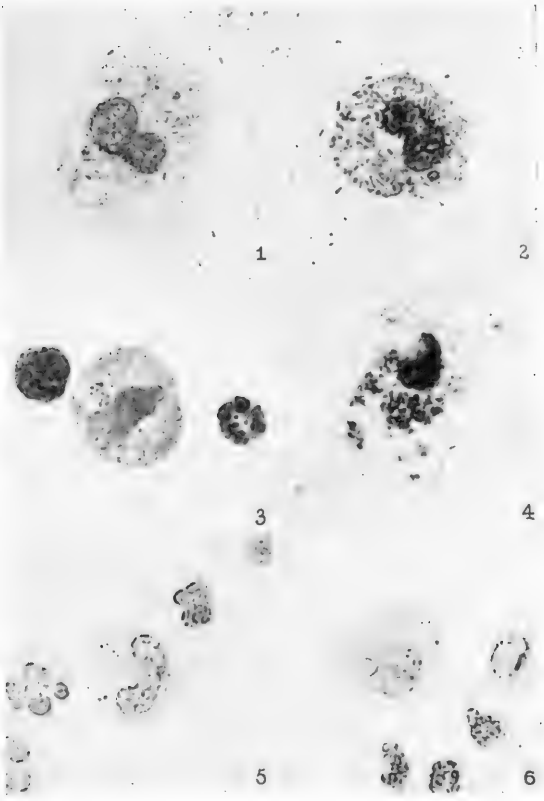
EXPLANATION OF PLATE 3.

Photomicrographs showing phagocytosis of bacteria by macrophages. Specimens (smears) were taken from oil-injected mice from 2 to 3 hours after they were inoculated with bacteria.

FIGS. 1 and 2. *B. coli.*

FIGS. 3 and 4. *Staphylococcus aureus.*

FIGS. 5 and 6. Pneumococcus.



(Nakahara: Macrophages in resistance to infection.)

AN EXPERIMENTAL STUDY OF THE RELATION OF THE OVARY TO FAT METABOLISM.

By MONTROSE T. BURROWS, M.D., and CHARLES G. JOHNSTON.

(*From the Research Laboratories of The Barnard Free Skin and Cancer Hospital, and the Department of Surgery of Washington University School of Medicine, St. Louis.*)

Plates 4 to 6.

(Received for publication, May 26, 1925.)

The controlling influence of the ovary on cyclic changes in the uterus and breasts has been fully appreciated for many years. Recently Allen and Doisy (1) have isolated from the follicular fluid of the ovaries of pigs an alcohol-, ether-, and acetone-soluble substance, which induces estrus when introduced subcutaneously into spayed rats.

It is also known that the ovary has to do with the metabolism of fat in the body. Women store fat after the removal of the ovaries, destruction of the ovaries from one of various causes, and after the menopause. Women suffering a premature loss of function of their ovaries also suffer various other metabolic changes and disturbances. In order to throw light on this latter property of the ovary it became of interest to study the action of the Allen-Doisy hormone in the digestion of fat in the tissues.[1]

In a previous study (2) we have shown that Mazola, or corn oil, which had been sterilized by heat, is not absorbed when injected into the subcutaneous tissue of most rats. In only one case out of thirty-nine was there any evidence of its absorption even after periods as great as 7 to 9 months (Fig. 3). This oil in the great majority of these cases simply breaks up into numerous small and larger droplets. Each of these droplets then becomes encapsulated by cells.

In this previous study we also described the method of the encapsulation of the oil droplets. These capsules are formed chiefly by large spherical shaped cells, having a single poorly staining nucleus. These cells are closely packed together at the edge of the oil droplet, forming a capsule one to several cells in thickness. Among these large cells are a few lymphocytes and neutrophils, and eosinophils, and polymorphonuclear and mononuclear leucocytes. The number of these

[1] Read before the Society of Experimental Pathology, Washington, D. C., December 29, 1924.

latter cells is never large and is the number that may be present at any time in blood vessels and tissue adjacent to the place where the oil was introduced.

This capsule is formed primarily of cells which migrate to the oil from the surrounding tissues. The large round cells are fibroblasts and endothelial cells, which have been drawn with the lymphocytes and leucocytes to the oil from the surrounding connective tissue and capillaries. None of the cells of the capsules have formed through proliferation. They have migrated to the edge of the droplets of oil from the tissue. During this migration the fixed tissue cells have not only rounded off to spherical shaped cells, but have suffered a loss in their ability to stain sharply.

This process of migration ceases after 48 hours. Subsequent to this time the fibroblasts and endothelial cells gradually regain their ability to stain sharply. They lay down intercellular fibrils, stretch out along the surface of these fibrils, and assume spindle shapes. In a few cases where large numbers of these cells had become crowded together at the edge of the droplets, the nuclei of the cells show an increase in chromatin. One division figure was seen in one specimen of this kind during this later recovery period. As a rule, however, the only evidence of growth is the formation of intercellular fibrils. Subsequently these cellular fibrous capsules then slowly regress to a hyaline scar (Fig. 3). The leucocytes and lymphocytes remain unchanged for a time in the capsule. Then they gradually disappear.

Corn oil does not stimulate a growth of cells. It acts to cause regressive rather than constructive changes in them. Evidences of growth about these oil droplets manifest themselves late, after the oil has ceased to attract the cells from the surrounding tissue and otherwise act upon them. This recuperation of the cells and growth are more and the amount of earlier degenerative changes in the cells is less about the smaller droplets than the larger ones.

Allen and Doisy had dissolved their hormone in corn oil in order to facilitate its introduction into the subcutaneous tissue. In each instance a tumor formed at the site of the injection. Since oil produces such tumors it had been thought that this reaction was due alone to the oil. In order to throw light on the mechanism of the absorption of the corn oil in one of our animals and not in the others, and in view of the relation of the ovary to fat metabolism in the organism, it became of interest to remove and study histologically a number of these tumors produced by the oil plus the ovarian hormone.

The animals used for these experiments were 60 rats, 2 guinea pigs, and a monkey. The hormone was prepared by first extracting the fresh follicular fluid

from the ovaries of pigs with alcohol, filtering, and evaporating the alcohol solution to dryness. The residue was then extracted with ether. After the ether solution was filtered it was evaporated and the residue extracted with the corn oil. This oil-hormone mixture was then introduced into the subcutaneous tissue of the animals.

The corn oil containing the hormone when introduced into the tissue breaks up into droplets the same as the pure corn oil. It excites likewise a rapid migration of cells to it from the surrounding tissues. A capsule forms which is identical with that seen about the pure oil except that the cells in migrating to the oil show very little evidence of degeneration, but rather an early active growth and division and an early and active laying down of intercellular fibrils. The cells also invade the oil, remove it, and proliferate most actively in the space occupied by it. Many of the smaller droplets of oil are completely removed by this process and the space originally occupied by them becomes a dense mass of proliferating fibroblasts. In the larger droplets this process ceases generally after a small amount of the oil is removed. After a given period of such proliferation this mass of new cells then slowly regresses and disappears in the form of a hyaline scar.

The details of this process are illustrated in the description of experiments below.

Experiment 1.—Two guinea pigs were injected with 1 cc. of corn oil containing the ovarian extract. The tumors were removed after 6 days. These tumors, like the tumors formed by injecting pure corn oil, are composed of numerous encapsulated droplets of the oil. Each of these droplets is surrounded by a layer of cuboidal cells. Outside this layer are layers of fibroblasts which at this stage have laid down a considerable amount of intercellular material and have stretched out to a spindle shape. Mitoses are numerous not only in the layer of cells about the oil droplets (Fig. 1), but also in the layer of cells deeper in the capsule. In many places these inner capsular cells are proliferating rapidly, the oil is disappearing, and the space occupied by it is gradually being replaced by these proliferating cells (Fig. 2).

As shown by the later experiments this proliferation into the smaller droplets continues until the oil is entirely removed and its site replaced by a dense mass of proliferating cells.

Experiment 2.—A rat was injected with 2 cc. of Mazola oil containing ovarian extract. The tumor was removed after 3 weeks. About all of the oil cysts the cells are seen proliferating actively. Many of the smaller drops of oil have been

entirely removed and replaced by these growing cells (Fig. 4, *A*). In other cases the cells are seen to be growing and slowly invading the oil from all sides (Fig. 4, *B*). The larger droplets of oil are still present (Fig. 4, *C*). Their former single layer of lining cells is seen now, however, as a layer many cells in thickness. About the edge of these larger oil droplets cell processes can also be seen projecting out into the oil as they are seen in Fig. 4, *B*.

Experiment 3.—59 rats were injected with 1 to 5 cc. of corn oil plus the ovarian extract. The tumors were removed from time to time, between 1 week and 7 months thereafter. The sections of tissue removed within 1 month show pictures similar to that seen in the guinea pigs and rat of Experiments 1 and 2. Subsequent to this time the proliferating tissue slowly regresses. In the great majority of the cases only the smaller oil droplets are found to have been removed by the cells. In other sections all of the oil has been removed. Only the original fat of the subcutaneous tissue remains. This active proliferation of cells continues, however, for only about 2 to 3 weeks, when it ceases and regression takes place. The denser masses of cells which fill many of the small oil cavities lose their nuclei. Their cytoplasm unites with that of neighboring cells. It becomes more granular and stains less sharply (Fig. 5). In the lower power picture of these areas after 6 weeks to several months one finds a few oil drops remaining and other open spaces which resemble the lacunæ left by cholesterol crystals (Fig. 6). Finally this mass of cells which filled the oil space shrinks to appear as a single large cell containing no nucleus or one or more poorly staining nuclei. The surrounding tissue undergoes slow regression to a hyaline scar containing a few small oil droplets. This process of regression takes place more quickly in some animals than in others.

Experiment 4.—One spayed monkey received 1 cc. of the corn oil plus ovarian extract. The tumor was removed after 3 months. The oil has been completely removed from this tumor. The tumor is composed of a cellular mass regressing to a hyaline scar (Fig. 7). The open spaces shown in this figure are for the most part the fat droplets of the original tissue or secondary fat deposits in the regressing scar.

DISCUSSION AND CONCLUSIONS.

From these observations there seemed to be little doubt that the follicular fluid of the ovary contains an active growth-stimulating substance and one capable of initiating an active digestion of a foreign fat, which might otherwise remain unabsorbed for an indefinite time in the tissues of these animals (2).

We have not attempted to ascertain whether this substance exciting growth and a digestion of the oil is the same or in any way related to the substance exciting estrus in these animals. That it may be a different substance from the estrus-exciting substance is suggested,

however, by the fact that a similar excitant of growth and fat digestion has recently been extracted by the same method from the corpus luteum of pigs. These extracts of corpora lutea have not excited estrus in spayed rats.

In the one rat in which the pure oil was absorbed, the cells did not invade the oil, but the capsule remained cellular and the oil gradually disappeared from the space. In these experiments in which the active substance was added to the oil the cells have always invaded the oil.

BIBLIOGRAPHY.

1. Allen, E., Francis, B. F., Robertson, L. L., Colgate, C. E., Johnston, C. G., Doisy, E. A., Kountz, W. B., and Gibson, H. V., *Am. J. Anat.*, 1924–25, xxxiv, 133. Doisy, E. A., Ralls, J. O., Allen, E., and Johnston, C. G., *J. Biol. Chem.*, 1924, lxi, 711.
2. Burrows, M. T., and Johnston, C. G., *Arch. Int. Med.*, 1925 (in press).

EXPLANATION OF PLATES.

PLATE 4.

FIG. 1. A moderately high power photomicrograph of a part of a section of a 6 day old tumor of a guinea pig formed by injecting corn oil plus ovarian hormone.

FIG. 2. A moderately high power photomicrograph of another part of the section shown in Fig. 1.

PLATE 5.

FIG. 3. A high power photomicrograph of a part of a section of an 8 months old tumor of a rat produced by the injection of 6 cc. of pure corn oil into the subcutaneous tissue. This picture is included to facilitate comparison between the action of pure oil and oil plus ovarian hormone.

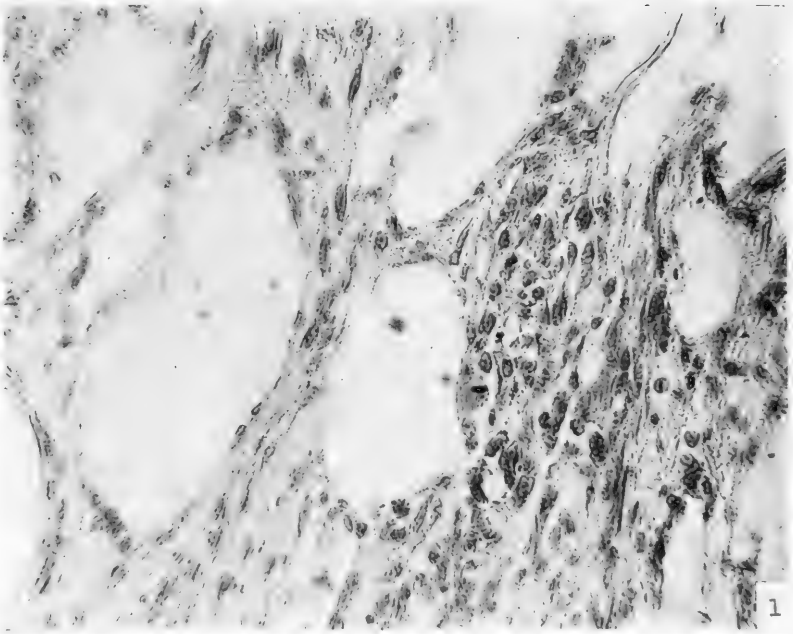
FIG. 4. A moderately high power photomicrograph of a part of a 3 weeks old tumor of a rat produced by injecting corn oil plus ovarian hormone.

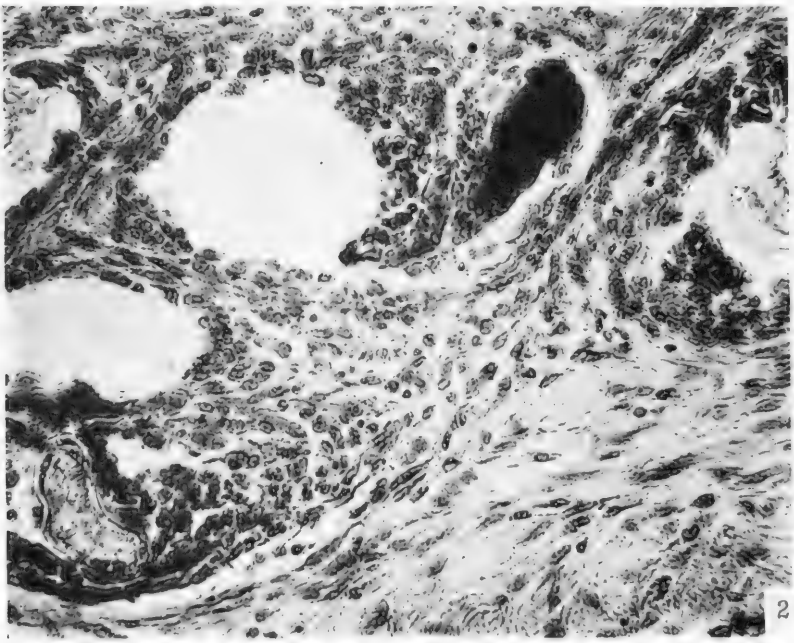
PLATE 6.

FIG. 5. A moderately high power photomicrograph of a section of a 7 months old tumor of a rat produced by injecting corn oil and ovarian hormone.

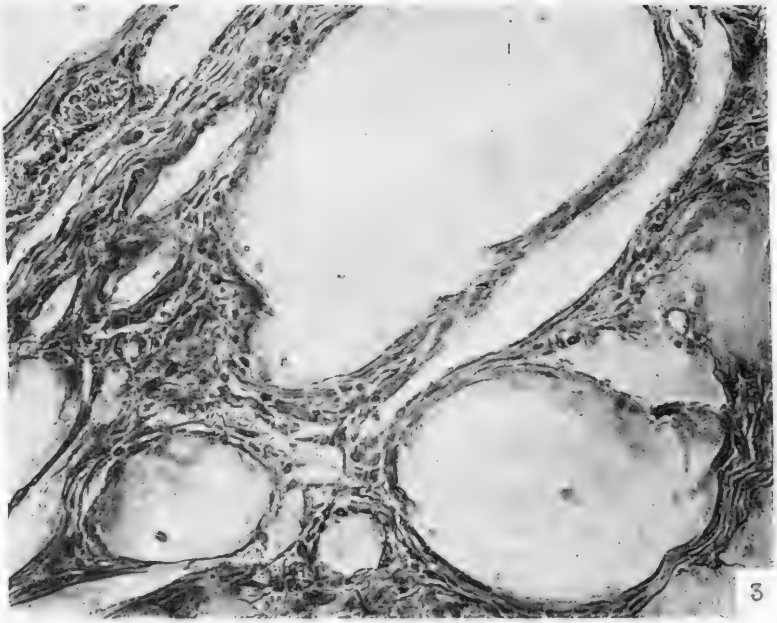
FIG. 6. A low power photomicrograph of the section shown in Fig. 5.

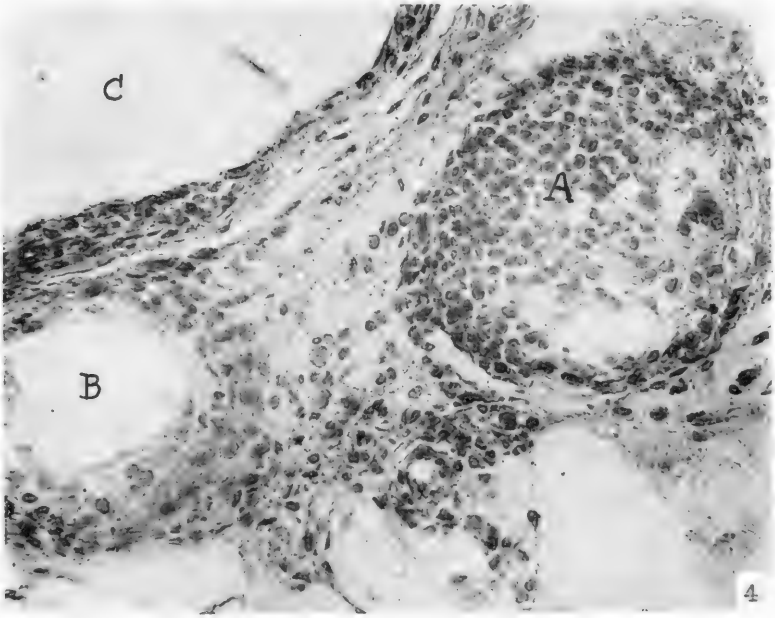
FIG. 7. A high power photomicrograph of a 3 months old tumor of a monkey produced by injecting 3 cc. of corn oil plus ovarian hormone.



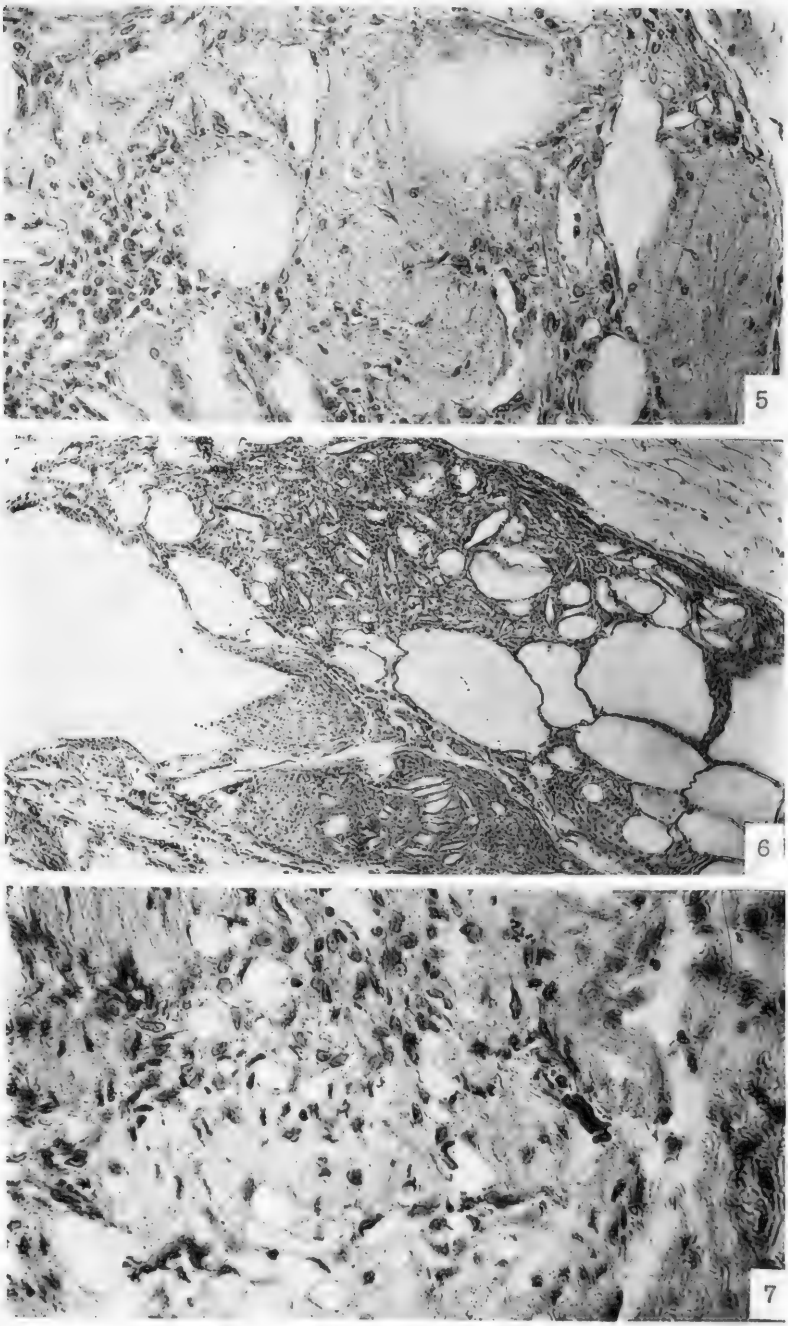


(Burrows and Johnston: Relation of ovary to fat metabolism.)





(Burrows and Johnston: Relation of ovary to fat metabolism.)



(Burrows and Johnston: Relation of ovary to fat metabolism.)

THE RELATION OF THE VITAMINS TO THE REACTION INDUCED BY COAL TAR IN THE TISSUES OF ANIMALS.

By LOUIS H. JORSTAD, M.D.

(From the Research Laboratories of The Barnard Free Skin and Cancer Hospital, and the Department of Surgery of Washington University School of Medicine, St. Louis.)

PLATES 7 TO 9.

(Received for publication, May 26, 1925.)

As the important problem in the study of cancer is the nature of conditions which regulate the normal growth of cells in the organism, it becomes necessary to study, in relation to tissue changes brought about by coal tar, the conditions and substances which promote normal growth. In previous papers (1) I have studied the action of coal tar in adult and embryonic tissues of the rat. These animals were fed on a balanced dietary throughout the experiments. Following the same procedure and technique, in so far as the introduction of coal tar into the tissue is concerned, I have modified the dietary in the feeding of a series of rats and studied the tissue changes produced by coal tar as affected by these conditions.

In the previous paper it had been shown that the chief effect of coal tar on the cells and organism is destructive rather than constructive. When drops of coal tar are injected into the subcutaneous tissue they attract the tissue cells to them from a wide area about them. As these cells leave the blood vessels and intercellular fibrils of the connective tissue the intercellular substances suffer not only a loss of their cells, but hyaline-like changes. The cells as they migrate to the edge of the tar round off, their nuclei and cytoplasm stain less sharply. Many of the first cells to arrive suffer a complete granular degeneration.

When a sufficient quantity of coal tar is injected into the subcutaneous tissue of an animal, the cells not only undergo this migration and show these degenerative changes, but the whole animal suffers a loss of weight, cachexia-like changes, and often an eventual death. The changes occurring in these animals as they are seen in the gross, are not unlike those that occur in animals with rapidly growing malignant tumors. Many of the earlier authors had already noted that lipoid solvents act to cause a death of the cells rather than any stimulation of them.

Such observations are seen in the articles by Bullock and Rohdenburg (2) and Champy (3). These authors had thought, therefore, that the action of these substances in the production of cancer is largely one of destruction with secondary regeneration. How the substances cause the degeneration and effect the general nutrition of the animal had not been determined, however.

The action of a single drop of coal tar is always limited. It acts to produce the changes noted above for only a short period of time. Its action is greatest at first, then it gradually wanes. It acts, therefore, in the tissue like a substance which dissolves some important constituent of the cell. Those cells which are drawn to the edge of these droplets of tar surround it as a continuous cellular layer one or many cells thick. After the tar has ceased to be active those of the cells which are not completely destroyed become active again. Their nuclei and cytoplasm stain more sharply. In the sparsely cellular tissue of adults a single injection of coal tar rarely ever draws more than a few cells to its periphery. In the more densely cellular mesenchyme of embryonic tissue and near the epidermis of adults many more cells are attracted. Where a large number of cells are crowded together about the tar they show growth and multiply as they recover from the primary effects of tar. Where only a few are attracted to the edge of the drop, they never show any growth, but slowly develop new intercellular substances and the whole eventually reverts to a hyaline scar.

The cells that move to the edge of the coal tar are the cells which are present in the tissues immediately about it. They are endothelial cells, fibroblasts, epithelial cells, leucocytes, and other wandering cells. With each new addition of coal tar more and more cells are attracted to the tar. With these repeated applications of tar the tissue about suffers much more exaggerated changes. Most or all of the cells in the nearby tissues are drawn to the tar. The surrounding tissues assume a hyaline appearance peculiar to tissue about actively growing cancers. In the more densely cellular masses accumulated by frequent applications of tar, growth and mitosis of cells intervene and cancer develops.

In studying these various effects of coal tar, it has been interesting to note that different animals respond differently to the injection of this substance. In a number of cases all the cells degenerated when they moved to the tar. In other cases they withstood the action of the tar and formed a dense cellular collar about each drop of this substance. Other authors have noted similar variations in these cellular responses in that cancer developed more rapidly in some of their animals, but no one has explained these variations. It became of interest, therefore, to investigate them in regard to the general state of nutrition of the animals and the diet they received.

For all these experiments young healthy actively growing animals have been used. The diets given them were modifications of those used by former workers on vitamins—Goldblatt (4), Gross (5), Zilva (6), Hume and Smith (7), Bond (8), Eddy (9), and McCollum (10). It was important to note carefully if disturbances

in nutrition and the arrest or retardation of growth was due to vitamin deficiencies in these diets, or due to non-palatability, or decreased consumption of food. Results due merely to lack of food intake were discarded.

The chief immediate index to the effect of any particular diet is the gain or loss in weight of the animal. Repeated weighings were carried out during the course of the experiment. The injections of coal tar into the animal were regulated as to time and amount on the basis of the weight curves and the study of tissue changes, after determining the effect of similar quantities of tar in a series of animals fed on an ordinary normal diet.

Diets.

1. Balanced ration.
   - Egg albumin scales (finely ground) ..... 25 gm.
   - Potato starch ..... 40 "
   - Butter ..... 15 "
   - Salt mixture (McCollum) ..... 5 "
   - Lemon juice ..... 5 cc.
   - Vegex* ..... 5 gm.
2. Moderately high in vitamin A.
   - Egg albumin scales ..... 25 gm.
   - Potato starch ..... 40 "
   - Butter ..... 25 "
   - Salt mixture ..... 5 "
   - Lemon juice ..... 5 cc.
   - Vegex ..... 5 gm.

2, *a*. High in vitamin A.
   Cod liver oil, 5 cc., added to (2).

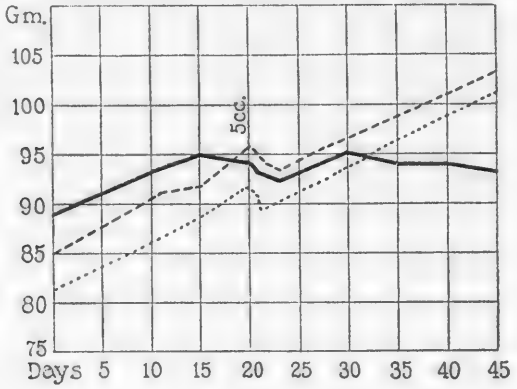
3. Deficient in vitamin A.
   Crisco substituted for butter in (2).
4. High in vitamin B.
   - Egg albumin scales ..... 25 gm.
   - Potato starch ..... 40 "
   - Butter ..... 15 "
   - Salt mixture ..... 5 "
   - Lemon juice ..... 5 cc.
   - Vegex ..... 10 gm.
5. Deficient in vitamin B.
   Vegex not included in the ration (4).
6. High in vitamin C.
   - Egg albumin scales ..... 25 gm.
   - Potato starch ..... 40 "
   - Butter ..... 15 "
   - Salt mixture ..... 5 "
   - Vegex ..... 5 "
   - Lemon juice ..... 10 cc.
7. Deficient in vitamin C.
   Lemon juice not added to the ration (6).

* Vegex is the source of vitamin B and a product furnished by the vitamin Food Company, Inc., Westfield, Mass.

EXPERIMENTAL DATA.

150 white rats were used in this experiment. They were divided into series, each series being made up of animals of practically the same age and weight. All were from the regular brother and sister strains of stock that has been bred in our laboratory during the past 5 years.

For the study of tissue changes around the particles of coal tar introduced under the skin, animals from each series were killed and autopsied at regular intervals of 2, 15, 30, 45, and 60 days after the injections of tar. A number of animals were kept for a longer period of time and some were autopsied and sections made of the tumor when the animal died as result of the deficiency diet. In other cases biop-



----------High vitamin B diet.
——————Low vitamin B diet.
.....Balanced diet.

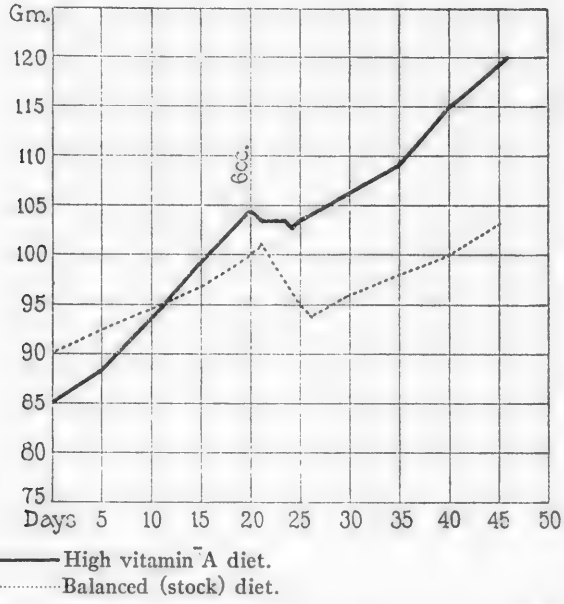
TEXT-FIG. 1. Curve showing changes in weight before and after the injection of 5 cc. of coal tar into three rats on a high vitamin B, deficient vitamin B, and balanced diet respectively.

sies were made from animals under ether anesthesia. A certain number in each series will be kept for a long period of time and findings reported later.

Discussion of Weight Curves.

The weight curves are practically self-explanatory and need emphasis only in regard to a few points of particular interest. Taking into

consideration the weight of the animal and the amount of coal tar injected over a period of time, the weight curves of the animals on a deficient vitamin B and a high vitamin B diet are practically identical (Text-fig. 1). There is a definite decrease in weight after each injection of coal tar and the composite growth curve follows a line almost parallel to the base line. The animals on a balanced dietary lost



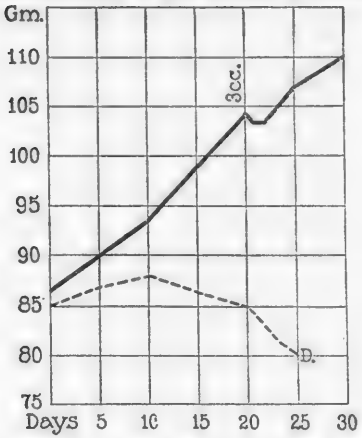
TEXT-FIG. 2. Curve showing changes in weight before and after the injection of 6 cc. of coal tar into two rats on a high vitamin A and a stock (balanced) diet respectively.

weight after each injection of coal tar, but they increased in weight gradually, not as rapidly, however, as an animal that has not been injected with coal tar (Text-fig. 2).

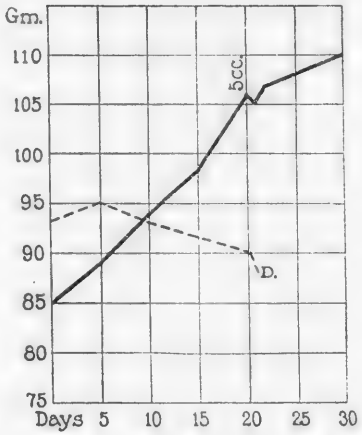
Animals fed the deficient vitamin A diet lost weight rapidly after the injection of coal tar, even after as small amount as 1 cc., and when the amount was increased to 3 cc., the animal died within a few days or even earlier. On the other hand, in the series receiving a high

vitamin A ration the weight curve was little affected by the injections (Text-figs. 3 and 4). 10 cc. of coal tar did not always kill these animals.

A deficiency in vitamin C gives a weight curve similar to that of a balanced dietary. The withdrawal of this vitamin has no effect on the reaction of the animal to the tar.



TEXT-FIG. 3.



TEXT-FIG. 4.

——————High vitamin A diet.
– – – – – – – –Low vitamin A diet.

TEXT-FIG. 3. Curve showing changes in weight before and after the injection of 3 cc. of coal tar into two rats on a high vitamin A and a low vitamin A diet respectively. Rats on a low vitamin A diet died (D.) quickly after injection.

TEXT-FIG. 4. Curve showing changes in weight before and after the injection of 5 cc. of coal tar into two rats on a high vitamin A and low vitamin A diet respectively.

Discussion of Tissue Changes.

In a previous paper (1) I discussed the tissue changes brought about by the introduction of coal tar into adult and embryonic tissue. The series of animals on the balanced ration dietary in this experiment presented tissue changes of the same character. There may be an earlier cellular degeneration and hyalinization in the tumors taken from the animals fed on the diet deficient in vitamin B but it is not marked. There is no cell activity after the 10 day period (Fig. 1).

In the animals fed on a high vitamin B diet, the collar of fixed tissue about the coal tar is more dense. Cells have been attracted from a

wider zone than in the case of animals fed on a balanced ration or one deficient in vitamin B. Activity is not marked, but the cellular content of the collar is greater than can be accounted for by a mere drawing in of cells from the surrounding tissue. From 15 to 20 days after the injection of tar, degeneration and hyalinization have taken place (Fig. 2).

The striking feature of the tissue changes brought about in the deficient vitamin A animals is the sparcity of cells in the zone about the coal tar. The cells have been attracted from the surrounding tissue, but these cells have suffered a marked degeneration in their migration to the tar (Fig. 3). In only one case did they show even slight activity. All of them shrivel and break up at an early period.

In the animals fed on the normal diet of the laboratory, on the specially prepared balanced diet, and one deficient in vitamin A or B, or high in vitamin B, the coal tar tends to remain in large drops as it was injected into the tissue. It is quite different in this regard from the corn oil described by Burrows and Johnston (11). In most cases the corn oil breaks up quickly into numerous small droplets.

In the animals fed on a diet high in vitamin A this picture changes. The coal tar breaks up quickly into a large number of small droplets (Figs. 4 and 6). These are found scattered in a densely cellular tissue. In a few of the sections removed from animals fed a balanced ration the periphery of the drop of coal tar is partially broken up by the cells. The picture is quite different, however, from the complete breaking up of the drop in the animals fed a diet high in vitamin A. The cells about these droplets are large and contain well formed, sharply staining nuclei. Many of them are dividing by mitosis. This picture is most marked in the 15 to 30 day specimen (Fig. 5). After this period certain portions of the tissue surrounding the droplets undergo hyalinization, cellular activity continuing only in scattered cell nests. These cell nests present the picture of early sarcomatous changes. The collars of cells about the individual droplets are much larger than in animals fed on other diets. They are often thirty cells thick.

DISCUSSION.

In my previous paper (1) I had shown that coal tar acts on the organism to cause cachexia-like changes. Drops of coal tar placed in the

tissue attract the fixed tissue cells to them from the surrounding tissues. They do not stimulate these cells to grow but the cells suffer partial or complete degeneration in this reaction. This action of the tar is not continuous, but limited to a short period of time. After the tar has ceased to be active those cells which have not degenerated completely recover from the action of the tar. Where the cells have become densely packed together about the tar active growth and division of these cells intervene and cancer develops. Where only a few cells are collected by the tar or only a few survive its action they form only a hyaline scar. There was no evidence that the tar acted, at any time, to stimulate the cells to grow. It acts as a simple solvent of lipoid elements in the cell. The degeneration in the cells and the cachexia may be the direct result of the removal of these lipoids by the tar.

Burrows (12) in his studies of body cells in the tissue culture has shown that these cells have many features in common with yeast and other unicellular organisms. They can grow independently as in cancer only when they are crowded together in a stagnant area where certain primary products of their metabolism can accumulate about them. They differ from many of the other unicellular organisms in that they have no special mechanism for migration. They migrate by liberating a surface tension-lowering substance absorbed only by proteins and fats. They can move only into proteins and towards larger droplets of fat which absorb this surface tension-lowering substance. The primary substance which must concentrate about these cells for them to become active is a water-soluble substance formed by them in proportion to the oxygen absorbed. It has been called the *archusia* (S). The surface tension-lowering substance has been called the *ergusia*. This latter substance is not liberated by the cell under all conditions, but only when the archusia (S) reaches a certain concentration (S_2). It is not re-formed in the cell until the archusia reaches a still higher concentration (S_3) or when the cells grow and divide. Coal tar dissolves the ergusia.

Body cells cannot retain the archusia. They can grow only when it is extracted from other sources and added to the medium or when they are crowded together in a stagnant medium from which it cannot escape. In the normal organism the archusia does not reach a con-

centration consistent for growth and re-formation of the ergusia. The question arose as to how these cells in the normal organism survive under such conditions. Is their mechanism for growth different from that in the culture, or do they obtain a supply of these substances from other sources? It seemed possible that certain of the vitamins were none other than these two essentials supplied by other growing animals and plants. To test this conception it became of interest to see the effect of coal tar on animals fed on a diet rich in one or the other of the various vitamins. These studies have shown that a diet rich in the water-soluble vitamin B stimulates the growth of these cells just as the archusia stimulates the cells in the culture, while the fat-soluble vitamin A protects the animal and the cells against the destructive action of coal tar or the loss of the ergusia to it.

In the light of these facts it has been possible, therefore, to draw the following conclusions.

SUMMARY AND CONCLUSIONS.

Drops of coal tar introduced into the subcutaneous tissue attract the fibroblasts, endothelial and other cells to them. These cells suffer degenerative changes through this action of the tar and the animal suffers cachectic-like changes and death from large doses of it introduced into the subcutaneous tissue. This action of the coal tar is limited to a short period of time, after which it becomes inert. The cells which have been drawn to it and which have not completely degenerated then slowly recover. Where large numbers of these cells are drawn to the tar they grow and divide after recovering from the initial effects of the tar. Cancer may develop. Where only a few cells are drawn to the tar they lay down intercellular fibrils and a scar eventually develops. Vitamin A fed in more than ample quantities to these animals protects the animals and the cells against the toxic action of the tar and stimulates and prolongs their secondary growth. Vitamin B stimulates the secondary growth of these cells. This action is limited in extent and time. It is followed by an early degeneration and hyalinization of the tissue.

BIBLIOGRAPHY.

1. Jorstad, L. H., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 67; *J. Cancer Research*, 1925, ix (in press).
2. Bullock, F. D., and Rohdenburg, G. L., *J. Med. Research*, 1915–16, xxxiii, 53.
3. Champy, C., and Vasiliu, I., *Bull. Assn. franç étude cancer*, 1923, xii, 111.
4. Goldblatt, H., *Biochem. J.*, 1923, xvii, 298.
5. Gross, L., *Biochem. J.*, 1923, xvii, 569.
6. Zilva, S. S., *Biochem. J.*, 1923, xvii, 410.
7. Hume, E. M., and Smith, H. H., *Biochem. J.*, 1923, xvii, 364.
8. Bond, M., *Biochem. J.*, 1922, xvi, 479.
9. Eddy, W. H., The vitamin manual, Baltimore, 1921.
10. McCollum, E. V., The newer knowledge of nutrition, New York, 2nd edition, 1922.
11. Burrows, M. T., and Johnston, C. G., *Arch. Int. Med.*, 1925 (in press).
12. Burrows, M. T., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 241.

EXPLANATION OF PLATES.

The figures are photomicrographs of sections of tissue of adult rats, showing the cellular changes about drops of coal tar. Each series of animals was placed on the different diets 30 days before the injection of the tar or oil.

PLATE 7.

FIG. 1. Moderately low power photomicrograph showing tissue changes 30 days after the injection of coal tar into the subcutaneous tissue of a rat fed on a diet deficient in vitamin B.

FIG. 2. Moderately low power photomicrograph showing tissue changes 30 days after the injection of coal tar into the subcutaneous tissue of a rat fed on a diet high in vitamin B.

PLATE 8.

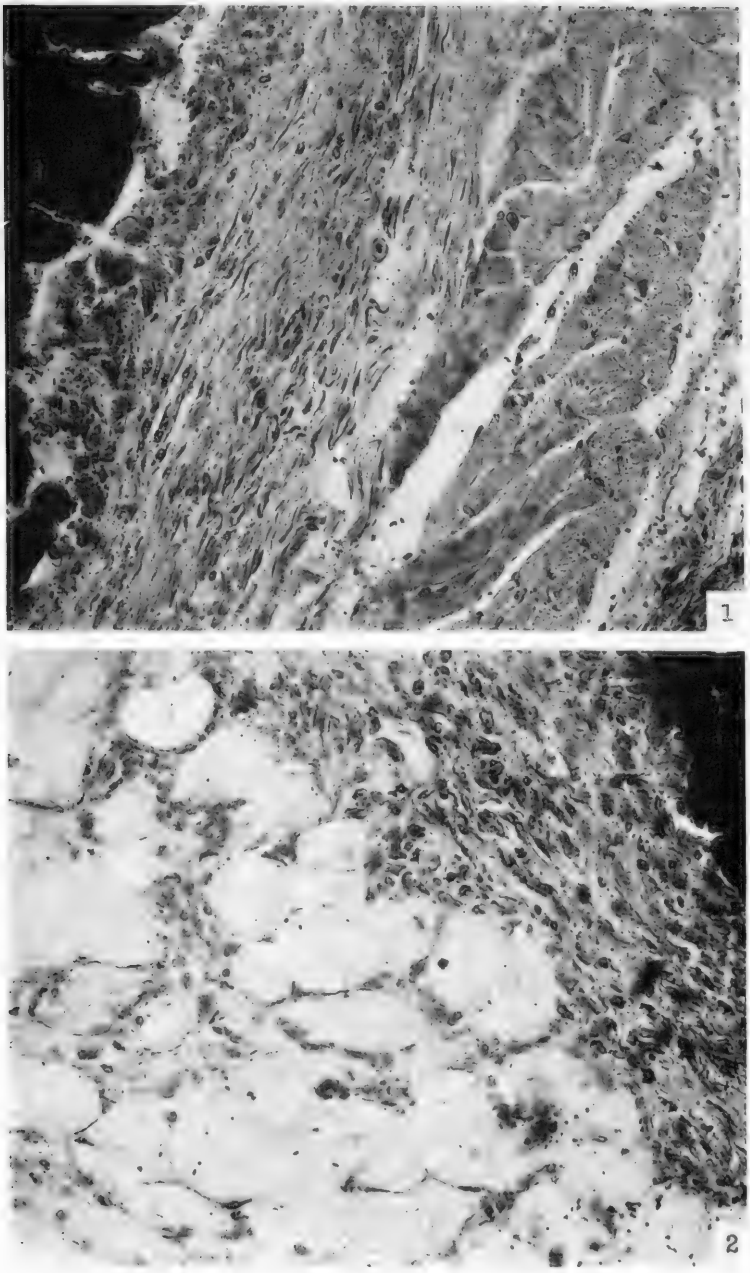
FIG. 3. Moderately low power photomicrograph showing tissue changes 28 days after the injection of coal tar into the subcutaneous tissue of a rat fed on a diet deficient in vitamin A.

FIG. 4. Moderately high power photomicrograph showing tissue changes 30 days after the injection of coal tar into the subcutaneous tissue of a rat fed on a diet high in vitamin A.

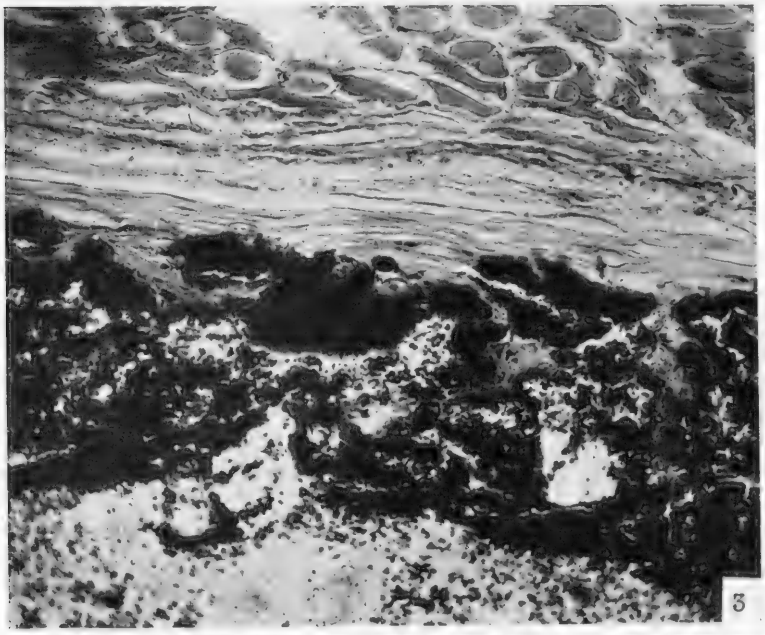
PLATE 9.

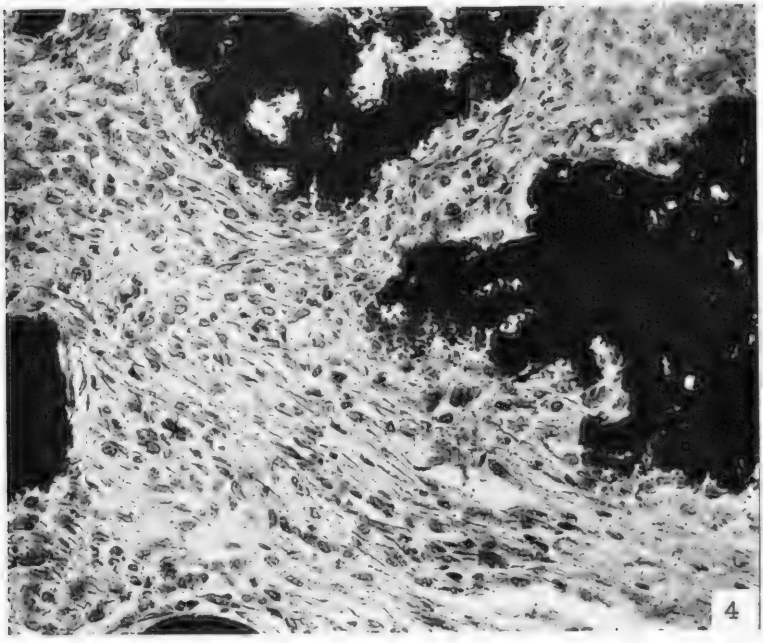
FIG. 5. A higher power photomicrograph of the same section as that shown in Fig. 4. The striking picture is the numerous mitotic figures as compared to sections taken from the rats on the other diets.

FIG. 6. A low power photomicrograph showing the breaking up of the coal tar into small droplets in the tissues of a rat on a high vitamin A diet. The section was taken from the rat 30 days after injection of the tar.

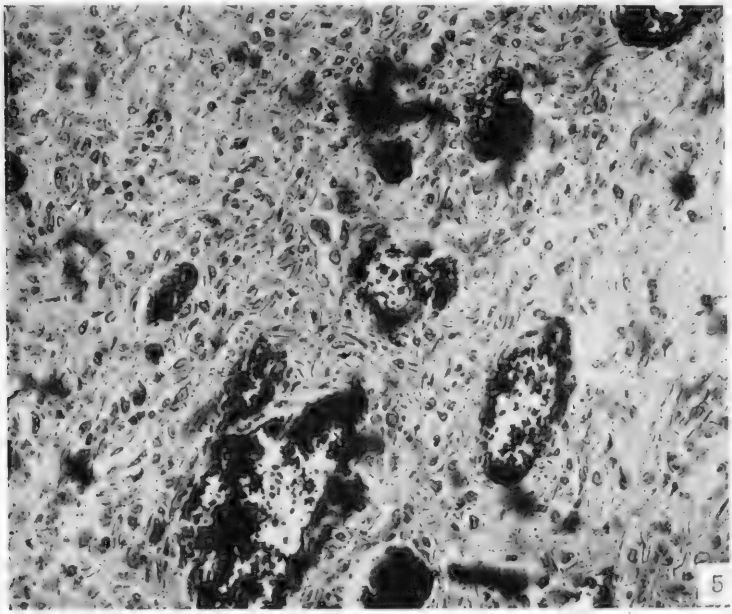


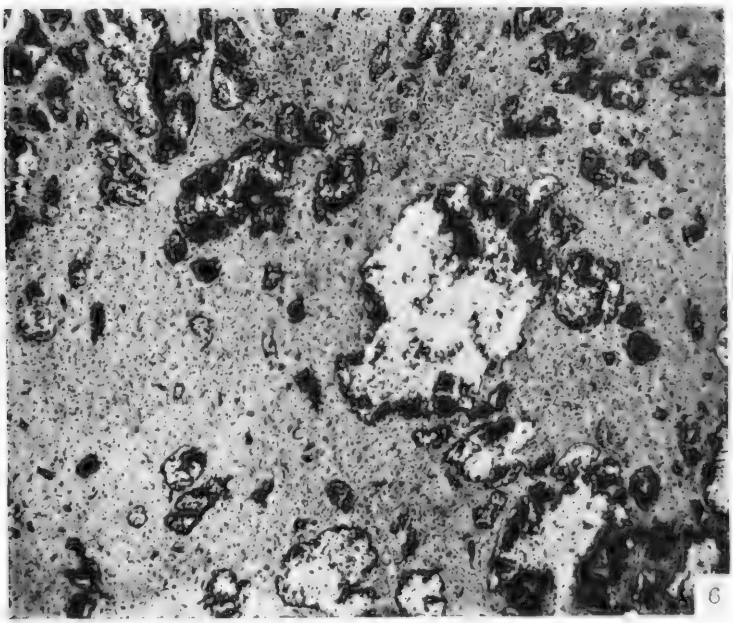
(Jorstad: Reaction induced by coal tar in tissues.)





(Jorstad: Reaction induced by coal tar in tissues.)





(Jorstad: Reaction induced by coal tar in tissues.)

STUDIES ON THE ETIOLOGY OF HEARTWATER.

I. Observation of a Rickettsia, Rickettsia ruminantium (N. Sp.), in the Tissues of Infected Animals.*

By E. V. COWDRY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 10 and 11.

(Received for publication, January 26, 1925.)

Heartwater is defined by Spreull[1] as "a specific febrile disease affecting sheep, goats and cattle in South Africa and due to an ultra-visible virus transmitted by the bont tick—*Amblyomma hebræum* (Koch)."

The name "heartwater" suggests the most characteristic lesion usually met with at autopsy; namely, hydropericardium. According to Hutcheon[2] the disease was first noticed in South Africa in 1860 coincident with the appearance of the bont tick, *Amblyomma hebræum*.[3] Owing to the severe economic losses which it has entailed it has since been the subject of much experimentation by Hutcheon[4] and others. Edington[5] was the first to transmit heartwater to susceptible cattle by the inoculation of blood containing the virus. In the same year, 1899, Louns-

---

* First contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made in the Laboratory of the Department of Agriculture at Onderstepoort and cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

[1] Spreull, J., *Agric. J. Cape of Good Hope*, 1904, xxiv, 433; *J. Agric. Union of South Africa*, 1922, iv, 236.

[2] Hutcheon, D., *Agric. J. Cape of Good Hope*, 1900, xvii, 410.

[3] Heartwater has since been reported in Angola and the Belgian Congo (Van Saceghem, R., *Bull. Soc. path. exot.*, 1918, xi, 423).

[4] Hutcheon, D., *Agric. J. Cape of Good Hope*, 1901, xix, 302; 1902, xx, 633; 1903, xxii, 438.

[5] Edington, A., *Agric. J. Cape of Good Hope*, 1904, xvii; *J. Comp. Path. and Therap.*, 1904, xvii, 141; *Rep. Director Gov. Bact. Inst., Grahamstown, Cape of Good Hope*, 1905, 27 (Colonial Secretary's Ministerial Division).

bury transmitted it to sheep and goats as also to cattle in 1902. He demonstrated[6] that normally the tick does not carry the virus, but that when it is allowed to feed upon a diseased animal it will pick up the virus, carry it through a moulting period (from larva to nymph or from nymph to adult), and transmit it to the next susceptible host. Lounsbury also discovered a most interesting fact, namely that a larva having obtained the virus and having fed as a nymph upon an insusceptible host is still capable as an adult of infecting its next host, if the host is susceptible. In this respect the virus differs from that of East Coast fever and also in so far that it is not inherited from one generation to another through the eggs.

The period of incubation is about 14 days when the disease is induced by ticks, somewhat shorter after blood inoculation. The febrile reaction is characteristic. The temperature usually rises abruptly to between 105° and 108°F. where it remains for several days, or as long as a week, and then often drops to subnormal before death. Occasionally there are nervous symptoms, such as muscular twitching, tetanic seizures, squinting, excessive salivation, and galloping movements after the animal has fallen to the ground. As Theiler[7] showed in 1903, the symptoms can only be explained on the supposition that we have to do with a microorganism present in the circulating blood. In his opinion the virus does not pass through a Berkefeld or Chamberland filter.[8] The mortality is high (over 50 per cent) and there is no satisfactory method of establishing protective immunity.

At the suggestion of Sir Arnold Theiler that the disease might be due to a *Rickettsia*, a cytological study of the tissues of experimentally infected animals was made in order to test this hypothesis by methods which have proved useful in the investigation of *Rickettsiæ*. It seemed possible that if this theory proved correct information might be secured which would have an interesting though indirect bearing upon the nature of Rocky Mountain spotted fever, typhus fever, and other diseases of man associated with *Rickettsiæ*, the viruses of

[6] Lounsbury, C. P., *Agric. J. Cape of Good Hope*, 1899; 1900, xvii, 682; 1902, xx, 29; xxi, 22, 165, 221, 315; *Rep. Gov. Entomol., 1903*, 15; *Agric. J. Cape of Good Hope*, 1904, xiv.

[7] Theiler, Sir Arnold, *Ann. Rep. Gov. Vet. Bact., 1903–04*, 114; *Vet. J.*, 1904, ix, 300; *Rep. Transvaal Dept. Agric., 1903–04*, 1905, 190; *Ann. Rep. Director Agric., 1904–05*, 1906, 121; *Ann. Rep. Gov. Vet. Bact., 1905–06*, 1907, 67; 1909, 33. Theiler, Sir Arnold, Gray, C. E., and Power, W. M., *10th Internat. Vet. Cong.*, London, 1914. Theiler, Sir Arnold, and Stockman, S., *J. Comp. Path. and Therap.*, 1905, xviii, 155.

[8] Knuth, P., and du Toit, P. J., Tropenkrankheiten der Haustiere, Leipsic, 1921, 628.

TABLE I.

Chart of Animal Passages.

Naturally infected goat from Pretoria district.

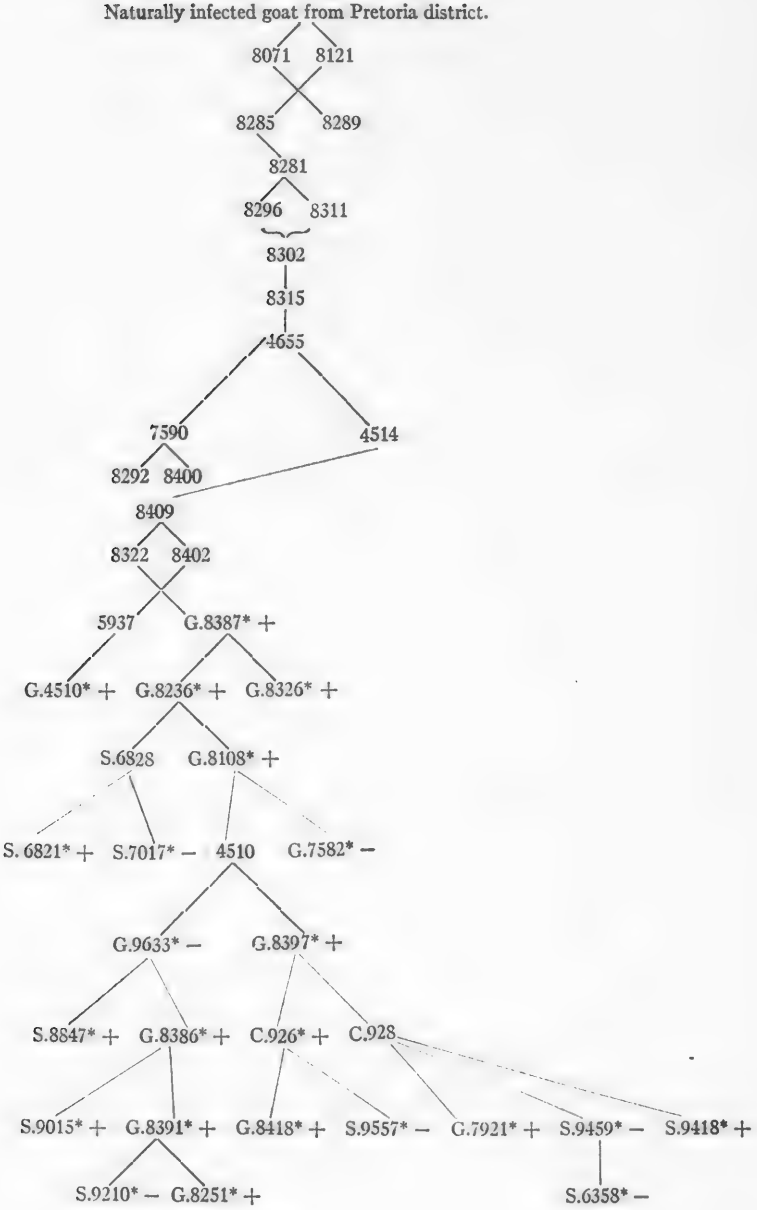


TABLE II.

Summary of Findings.

No.	Inoculated with.	Temperature began to rise on.	Attained maximum on.	Animal died or was killed on.	Pathological changes.	Distribution of microorganisms.
G. 4510	10 cc. blood from G. 5937, no reaction; 10 days later 10 cc. blood from G. 8108.		8th day. 106.8°F.	Killed 14th day. 103°F.	Hydrothorax; marked hydropericardium, fatty degeneration and pigmentation of liver; hyperemia of kidneys and slight enteritis.	Spleen, kidneys, cerebral cortex, cerebellar cortex, midbrain, suprarenal, pancreas, lymph gland, and heart muscle.
G. 7582	10 cc. blood from G. 8108.	7th day.	10th day. 108°F.	Killed 16th day. 105°F.	Anemia. Petechiæ in mucous membrane of trachea and bronchi. Subendocardial petechiæ. Slight hydropericardium and fatty infiltration of liver with pigmentation chiefly in center of the lobules. Tumor splenis. Erosions in mucous membrane of esophagus. Slight infestation with *Hæmonchus contortus*.	None observed.
G. 8108	50 cc. blood from G. 8236.	8th "	12th day. 107.2°F.	Killed 13th day. 107°F.	Hemorrhages under epicardium and left endocardium, emphysema and edema of lungs. Edema and pigmentation of liver (infiltration of interstitium and green pigmentation around central veins). Tumor splenis; interstitial nephritis; slight catarrhal gastroenteritis; larval cysts of *Tænia hydatigena* in omentum and mediastinum; *Trichuris ovis* in cecum.	Spleen, suprarenal, and lymph gland.

G. 8236	10 cc. blood from G. 8387.	11th day.	12th day. 108°F.	Killed 12th day. 105.6°F.	Fibrous pericardial adhesions; tumor splenis; hyperemia of kidneys; stasis and pigmentation of liver; hyperemia of lungs; wireworms in stomach; enteritis; parasitic nodules in omentum; subendocardial hemorrhages in left ventricle.	Spleen.
G. 8326	10 cc. blood from G. 8387.	11th "	12th day. 106.4°F.	Killed 13th day. 105°F.	Slight hydrothorax; edema of lungs; pigmentation and enlargement of liver; degeneration of myocardium and kidneys.	Cerebral cortex.
G. 8386	10 cc. blood from G. 9633.	10th "	13th day. 106.6°F.	Killed 13th day. 106.6°F.	Edema of lungs; areas of fibrosis in liver capsule as a result of perihepatitis chronica; tumor splenis; nephritis (?); slight hydropericardium; caseous lymphadenitis of mediastinal lymph glands; helminthiasis nodularis chiefly in large intestine.	Kidney, cerebral cortex, midbrain, suprarenal, and lymph gland.
G. 8387	10 cc. blood from G. 8322, G. 8402.	7th "	10th day. 107°F.	Killed 12th day. 105°F.	Hydropericardium; epicarditis fibrosa; edema of lungs with marked interstitial edema; chronic cholangitis of intrahepatic bile ducts; *Stilesia hepatica* in the lumina; tumor splenis; fatty infiltration of kidneys; few *Hæmonchi contorti* in abomasum; few foci of coccidiosis and *Œsophagostomum columbianum* nodules in intestine. 2 larval cysts of *Tænia hydatigena* attached to serosa of bladder.	Cerebral cortex.

TABLE II—*Continued.*

No.	Inoculated with.	Temperature began to rise on.	Attained maximum on.	Animal died or was killed on.	Pathological changes.	Distribution of microorganisms.
G. 8391	10 cc. blood from G. 8386.	10th day.	11th day. 107.6°F.; excised lymph gland under chloral hydrate.	Died 17th day. 100°F.	Interval after death 7–10 hrs.; hypercemia of lungs; edema and emphysema of right auricle and left endocardium; marked tumor splenis.	Kidney.
G. 8397	10 cc. blood from G. 4510.	10th "	12th day. 106°F.	Killed 12th day. 106°F.	Acute anemia (due to bleeding); slight hydropericardium; degenerative changes in liver and kidneys; tumor splenis; slight nodular worm infestation.	Kidney, medulla oblongata, corpus luteum, and lymph gland.
G. 8418	10 cc. blood from C. 926.	11th "	12th day. 106.4°F.	Died 14th day. 104.2°F.	Interval 1½ hrs.; slight hydrothorax and hydropericardium; petechiæ in left ventricle; fibrous adhesions of liver and diaphragm; tumor splenis; parasitic nodules in large intestine.	Kidney and corpus luteum.
S. 8847	10 cc. blood from G. 9633.	11th "	12th day.	Killed 13th day. 106°F.	Slight hydropericardium; slight edema of lungs; few small calcareous nodules under the capsule; tumor splenis; edema of kidneys; slight infestation with *Hæmonchus contortus*.	Kidney.
G. 9633	10 cc. blood from G. 4510.	5th "	7th day. 107°F.	Killed 8th day. 105.8°F.	Hydropericardium; subendocardial hemorrhages; tumor splenis; wireworm infestation; old *Œsophagostomum* nodules.	None observed.

S. 6821	50 cc. blood from G. 5937.	13th day.	14th day. 106.8°F.	Killed 18th day. 105.6°F.	Slight hydrothorax; slight hyperemia and edema of lungs; infiltration and slight pigmentation of liver; parasitic nodules in liver; subserosal hemorrhages in cecum; *Œsophagostomum columbianum* nodules.	Spleen, kidney, cerebral cortex, and salivary gland.
S. 7017	10 cc. blood from S. 6828.	6th "	10th day. 107.6°F.	14th day normal. Animal recovered. Killed on 30th day.	Hydropericardium; general anemia; *Œsophagostomum columbianum* nodules.	None observed.
S. 9015	10 cc. blood from G. 8386.	10th "	13th day. 107°F.	Died 14th day.	Interval after death 6 hrs. Anemia; sutured wound in left flank; slight hydrothorax and hydropericardium; hypostasis of right lung; fair number of *Œsophagostomum columbianum* nodules in intestine; larval cysts of *Tænia hydatigena* in omentum.	Kidney.
C. 926	20 cc. blood from G. 8397.	11th "	13th day. 106°F.	Killed 14th day. 104°F.	Acute anemia due to bleeding; hydropericardium; edema of lungs; hyperemia of liver; tumor splenis.	Kidney and cerebral cortex.
G. 8251	10 cc. blood from G. 8391.	7th "	Killed 9th day. 103°F.		Slight stasis in lungs; ecchymoses in left endocardium.	Kidney (only a single clump).
G. 7921	10 cc. blood from C. 928.	8th "	12th day. 107°F.	Died 14th day. 103.2°F.	Hydropericardium; hydrothorax; ecchymoses under left endocardium; petechiæ and edema of lungs; stasis and fatty infiltration of liver; marked tumor splenis; slight gastritis.	Kidney, ovary, corpus luteum, cerebellar cortex, corpus striatum, cerebral cortex (organisms not well stained, particularly in kidney and corpus luteum).

TABLE II—*Concluded.*

No.	Inoculated with.	Temperature began to rise on.	Attained maximum on.	Animal died or was killed on.	Pathological changes.	Distribution of microorganisms.
S. 9210	10 cc. blood from G. 8391.	Killed 11th day. 104.8° F.			Slight lymphatic hyperplasia of spleen; *Œsophagostomum columbianum* nodules; *Hæmonchus contortus*.	None observed.
S. 9557	10 cc. blood from C. 926.	10th day.	13th day. 107°F.	18th day temperature normal. 24th day killed.	Parasitic nodules and fatty degeneration of liver; slight fatty degeneration of kidneys; *Œsophagostomum columbianum* nodules in intestine.	" "
S. 9459	10 cc. blood from C. 928.	8th "	11th day. 107.6°F.	17th day temperature normal. 20th day killed.	Hydropericardium; general anemia; infarcts in kidneys; parasitic nodules in intestines.	" "
S. 6358	20 cc. blood from S. 9459. 105° F. Temperature fell to 102° 2nd day.	12th "	Killed 13th day. 104°F.		Tumor splenis, hyperplasia of follicles in spleen; *Œsophagostomum columbianum* nodules; *Ecchinococcus granulosus* in lungs; *Stilesia hepatica* in bile ducts.	" "
S. 9418	20 cc. blood from C. 928.	11th "	15th day. 107.6°F.	Died or killed 17th day. 107°F.	Hydropericardium; slight ascites; degeneration of myocardium; tumor splenis; swelling and fatty infiltration of kidneys; edema of cecum; 2 *Hæmonchi contorti; Œsophagostomum columbianum* nodules; calcified cyst of *Cysticercus tenuicollis;* few foci of coccidiosis in small intestine.	Medulla oblongata and cerebellum.

which, like that of heartwater, have thus far resisted all attempts at artificial cultivation and of which arthropods are the vectors.

Transmission of the Disease.

The heartwater virus employed transmitted the disease with great constancy and was passed through many animals, as shown by the record given in Table I. For convenience each animal was designated by a laboratory number under which heading all information regarding it was assembled. An asterisk (*) indicates that histological examination was made, a plus sign (+) that microorganisms were found, and a negative sign (−) that microorganisms were not observed.

In making the passages infective blood was injected intrajugularly into goats (G.), sheep (S.), and cattle (C.). After a period of incubation of about 10 days the temperature rose suddenly to from 106–108°F. and then subsided, shortly after which the animals usually died or else were killed. Careful autopsies of all were made by the veterinary officers on duty (see Table II). For routine purposes tissues were fixed in Zenker's fluid and were colored by Giemsa's stain, but other special fixatives and stains were also employed as will be mentioned subsequently.

Controls.

The following controls were examined and proved negative for the microorganisms under discussion.

Three sheep and three goats which were held under careful observation for several days before they were killed in order to make certain that they exhibited no signs whatever of disease. For this material I am indebted to Dr. Steck. There were also examined twelve sheep which died from bleeding in the preparation of blue-tongue vaccine; four sheep killed in an advanced stage of jagsiekte;[9] two cattle which died of snotsiekte;[10] and a cow which died of lamsiekte.[11]

---

[9] Jagsiekte is a contagious chronic catarrhal pneumonia of sheep, the causative agent of which is unknown (Mitchell, D. T., *Rep. Director Vet. Education and Research* (Sir Arnold Theiler), 1915, 585).

[10] "Snotsiekte is an acute specific infectious disease of cattle caused by an ultra-microscopic but non-filterable organism and characterised by a general hyperplasia of lymphoid tissue throughout the body, less frequently by inflammation, erosion and necrosis of the various mucosæ" (Mettam, R. W. M., *Rep. Director Vet. Education and Research* (Sir Arnold Theiler), 1923, 393).

[11] Lamsiekte is a disease of cattle caused by poisoning through the ingestion of a toxin produced by "an anaerobic bacterium reminiscent of, but not identical with, *Bacillus botulinus*" (Theiler, Sir Arnold, Green, H. H., and du Toit, P. J., *J. Agric. Union of South Africa*, 1924, 4).

The Observation of Microorganisms in Animals Experimentally Infected with Heartwater.

At intervals during the febrile period fresh blood was examined by direct and oblique illumination and in smears stained in a variety of ways. No microorganisms were ever found, although it is known that the blood was infective at the time when procured. Fresh smears of various organs taken at autopsy were likewise examined without results. It was only in the fixed and stained tissues that peculiar Gram-negative cocci were found, first in the spleen and later in the other organs. In making the histological examinations it was often necessary to spend hours going over entire sections with the help of a mechanical stage, as is the case also in the demonstration of *Rickettsiæ* in the tissues of animals suffering from Rocky Mountain spotted fever.[12] For this purpose a 1.5 mm. apochromatic objective and a No. 8 compensating ocular were employed.

The microorganisms were most easily detected in the endothelial cells of the capillaries of the renal glomeruli (Figs. 3 and 11) and in the superficial gray matter of the cerebral cortex (Figs. 4, 6 to 8, 10, and 15), which latter, it will be remembered, is the location where Wolbach, Todd, and Palfrey[13] suggested that search be made for the *Rickettsiæ* of typhus fever in experimentally infected guinea pigs. The microorganisms were also found in the following tissues in order of frequency: spleen (Figs. 1 and 2), lymph glands, corpus luteum, cerebellar cortex, suprarenals, midbrain, medulla oblongata, ovaries, corpus striatum, salivary glands, pancreas, and heart muscle. Since they were never found in either the liver or the lungs, it is safe to conclude that in these organs they are either of very rare occurrence or wholly absent. Testicles were seldom available for examination as most of the males had been castrated.

The observation of the microorganisms in the tissues proved to be a rapid and inexpensive method of diagnosis in the case of sheep suffering from anaplasmosis but dying from superimposed attacks of heartwater.

[12] Nicholson, F. M., *J. Exp. Med.*, 1923, xxxvii, 221.

[13] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

It was found that after death the microorganisms remained in the tissues for at least 6 hours. In the case of Sheep 9015 (Table II) they were demonstrated in the kidneys after this length of time, but with considerable difficulty since they had lost their affinity for basic dyes and were recognizable only by their position and morphology. Microorganisms were also detected in fragments of spleen taken from this sheep and kept for a further interval of 11½ hours at room temperature, but there also they were seldom of typical appearance. This early change in the microorganisms runs parallel with the rapid loss of infectivity of the blood and tissues after death.

The Association of the Microorganisms with the Febrile Reaction and with the Lesions.

A very close correspondence was noted between the presence of microorganisms and the febrile reaction. They were never seen in the incubation period, but invaded the endothelial cells before the maximum temperature was reached and as early as 2 days after the initial rise in temperature. It was found that the most favorable time to search for them was when the temperature was subsiding, from 2 to 4 days after the maximum temperature had been reached. They usually persisted, however, for a period of 6 days at the end of which time the temperature had generally fallen to normal. Failure to observe them later than 6 days after the time of maximum temperature was in general accordance with the loss in infectivity of the blood when inoculated into susceptible animals.

Goat 9633 seems to have been an exception in that no microorganisms were found in its tissues although it was killed on the 1st day of the decline in temperature. The incubation period in this case was atypical, being reduced to 5 days, but there was every reason to believe that the animal was actually suffering from heartwater.

The most characteristic lesion and one invariably associated with the presence of the microorganisms was a marked swelling of the endothelial cells. They were seen within the endothelial cells in dense masses varying from a few individuals up to several hundreds (Figs. 1 to 15). The clumps of microorganisms were always found to be surrounded by a halo of cytoplasm staining very lightly or not at all. Isolated microorganisms were never observed. The endo-

thelial enlargements were found in some cases to be so extensive as to entirely block up the lumina of the capillaries—a condition illustrated in Fig. 1. In other cases portions of the cells containing microorganisms became detached and passed into the circulation, or the cells ruptured, with discharge of microorganisms into the blood stream (Fig. 3). The fact, already mentioned, that attempts by many methods to detect the microorganisms in the circulating blood during the febrile reaction were unsuccessful may have been due to the splitting up of these masses of microorganisms into single individuals which, owing to their spherical form, would be difficult to identify in the living state. Moreover, in fixed preparations the stain would be very easily extracted during differentiation on account of their small size.

Except for the presence of the microorganisms the cytoplasm of the endothelial cells showed no deviation from the normal and it seldom contained recognizable products of the phagocytosis of hemoglobin or fatty inclusions, or any of the basophilic granulations reported in typhus fever.

Neighboring endothelial cells devoid of microorganisms appeared normal and were not enlarged. No evidence was found of undue multiplication of the endothelial cells. No thrombosis was noted and the microorganisms were never seen in association with any kind of leucocytic infiltration or in any extravascular location. In other words, they seemed to excite no detectable local tissue reaction other than the physical distention of the cells to accommodate them in large number.[14]

The Morphology of the Microorganisms.

The microorganisms were found to be very uniform coccus-shaped bodies, 0.2 to 0.5 μ in diameter, as measured after fixation in Zenker's fluid or Regaud's fluid and coloration by Giemsa's stain, or by any basic aniline dye. Their spherical shape is well shown in Figs. 1 to 6. No agglutinations or marked irregularities in morphology suggestive of intracellular digestion were observed. In a single clump the

[14] The histopathology of heartwater will soon be made the subject of a special paper by Dr. Steck, who has also enjoyed the privilege of working in Sir Arnold Theiler's laboratory.

microorganisms were, so far as could be seen, of the same size; but adjacent clumps in the same section occasionally differed slightly in the size of the individuals composing them, within the limits specified above and as represented in Fig. 1. The microorganisms had the appearance of being slightly larger when present in small masses and when not closely crowded together, as in the case of accumulations of many hundreds; but the difference was not sufficiently marked to exclude the possibility that it may have been merely an optical illusion. Occasionally they were observed in diplo formation (Fig. 11), but this was the exception rather than the rule. No morphological evidence was detected of any multiplicative phase, other than this diplo formation indicating the likelihood of simple division. The shape and size of the microorganisms remained constant throughout the febrile reaction.

Microchemical Reactions of the Microorganisms.

The microorganisms were well preserved after fixation in Regaud's fluid[15] and Zenker's fluid both with and without acetic acid, but all the fixatives commonly used for bacteria were suitable. They were colored deep, clear blue by Giemsa's method. Those in diplo formation showed no trace of red-staining material between the two halves such as has often been described in *Rickettsiæ* in lice; but sometimes they exhibited halos. They were likewise easily stained by Löffler's methylene blue and other basic aniline dyes. When treated with Unna-Pappenheim's methyl green-pyronine mixture, they usually stained light red, but sometimes acquired a slightly greenish tint, depending upon the method of staining. They were Gram-negative and stained readily with fuchsin, but did not retain it on differentiation. They were nicely stained with iron-hematoxylin, but on differentiation became bleached before the nuclear chromatin and erythrocytes. Some resisted decolorization more than their neighbors and remained jet-black while the others became light gray, but this may have been occasioned by irregular washing out of the mordant (iron alum) and may not indicate the existence of a true qualitative difference *inter se*. When autolysis of the tissue was

[15] 4 parts of a 3 per cent solution of potassium bichromate and 1 part of commercial formalin—a fixative intended primarily for the study of mitochondria.

allowed to proceed their affinity for basic dyes was lost before that of the nuclear chromatin.

Distinction from Normal Cellular Components and the Products of Degeneration and Phagocytosis.

Although the association of the microorganisms with heartwater, their morphology, and their staining reactions were found to be so definite, it was decided to compare them carefully with normal cellular components and with the products of degeneration and phagocytosis, since, up to the present time, like most *Rickettsiæ*, their status as living organisms has not been proved by methods of artificial cultivation.

When they were first detected in the spleen (Figs. 1 and 2) the possibility of confusion with some unfamiliar granular type of blood or bone marrow cell was carefully considered on account of their characteristic tendency to occur in dense clumps, but this was soon definitely excluded by the observations, already alluded to, that they seemed to be identical in three species (*i.e.* goats, sheep, and cattle); that they occurred only in a certain phase of the febrile reaction, being absent in controls; and that, in distribution, they were restricted to the cytoplasm of endothelial cells, being surrounded by a distinct zone of rarefaction. Another point of distinction was that the microorganisms were always disposed in clumps at one side of the nucleus. The nucleus was never surrounded on all sides by them as is usually the case with the specific granules in granular leucocytes. Although a search was made of the bone marrow, no cells containing granules at all resembling them were found.

Unlike mitochondria, they were readily preserved by Zenker's fluid containing the usual 5 per cent of acetic acid. They were basophilic, whereas the mitochondria are acidophilic. In shape also they were different from mitochondria, because no rod-like or filamentous forms were seen. They were present in the cytoplasm in large and compact masses in contrast to the mitochondria which are never so abundant and are not grouped in the same way. Finally, by their restriction to the endothelium, they were clearly differentiated from mitochondria and all mitochondrial products.[16]

The microorganisms were also distinguished from the granules of mast cells by their uniformity in size and shape, by the fact that they generally stained clear blue, instead of purple, by Giemsa's method, and similarly by their restriction

[16] The distinction between mitochondria and bacteria is discussed by Cowdry and Olitsky (Cowdry, E. V., and Olitsky, P. K., *J. Exp. Med.*, 1922, xxxvi, 521), and between mitochondria and the *Rickettsia* of Rocky Mountain spotted fever by Nicholson.[12]

to the cytoplasm of endothelial cells (see Fig. 5). They were never found outside the blood vessels.

Neither were the microorganisms to be confused with products of the phagocytosis of hemoglobin—an explanation of the nature of *Rickettsiæ* advanced by Woodcock.[17] In the first place, phagocytosis of fragments of red blood cells by the endothelium was not a process commonly met with in heartwater. In the rare instances in which it did occur the resultant inclusions took the form of droplets grading from about the size of a red blood corpuscle down to a few microns in diameter and stained much more strongly than did the microorganisms with iron-hematoxylin. When colored by Giemsa's method, these inclusions frequently exhibited a yellowish or greenish tinge, due to the superposition of pigment and stain. They were never clear blue like the microorganisms, nor were they clumped in so characteristic a fashion.

The microorganisms stained much the same color as nuclear chromatin, but were easily differentiated from it or any of its degenerative products. As is indicated by the figures, the nuclei of the endothelial cells containing them seldom revealed any traces of division or degeneration so that chromatin was not being emitted in a form detectable morphologically. It was to be noted that except for the presence of the microorganisms the cytoplasm of the endothelial cells showed no modifications microscopically visible. In this respect the condition of the vascular endothelium in heartwater differed sharply from that found in typhus fever as described by Wolbach and his associates.[13,18]

DISCUSSION.

It is interesting to compare the above mentioned microorganisms with *Rickettsiæ*, but to do so consistently is by no means a simple matter because there is so little unanimity of opinion as to what distinguishing features *Rickettsiæ* actually possess.

If the suggestion, made in an earlier paper,[19] that in the identification of *Rickettsiæ* stress may properly be placed upon "the ability of the organisms to lead an intracellular existence, their location in the tissues, their host specificity, their Gram-negative properties, and their bacterium-like morphology" be accepted by others, as it has been by Hertig and Wolbach,[20] we are clearly justified in including these microorganisms in the general category of *Rickettsia*. These authors, in practice have, however, slightly modified the definition to read as follows:

---

[17] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1921, xxxvii, 418; 1922, xxxix, 243; 1923, xl, 81, 241; 1924, xlii, 175.

[18] Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

[19] Cowdry, E. V., *J. Exp. Med.*, 1923, xxxvii, 431.

[20] Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1924, xliv, 329.

TABLE III.

Comparison of the Rickettsiæ of Rocky Mountain Spotted Fever and of Typhus Fever with the Microorganisms in Heartwater as Seen in Mammalian Tissues.

	Spotted fever.	Typhus fever.	Heartwater.
Morphology.	(*a*) Paired; 0.2 to 0.3 by 1 μ; often surrounded by halo. (*b*) Rod-like forms 1 μ in length and often possessed of polar granules. (*c*) Rounded forms.[23]	(*a*) Ovoid, somewhat lanceolate bodies in pairs. The pairs measure slightly over 1 by 0.2 to 0.3 μ. (*b*) Smaller coccoid bodies.[13]	Very uniform coccoid bodies 0.2 to 0.4 μ in diameter, rarely in pairs.
Phases of multiplication.	None detectable in mammalian tissues other than possibly simple division.	The same.	The same.
Microchemical reactions.	Best seen after fixation in Zenker's fluid and coloration by Giemsa's stain. Easily stained by basic aniline dyes, Gram-negative, and not acid-resistant.	The larger paired bodies stain more readily than the cocci and are surrounded by a slight halo.[13] Gram-negative and not acid-resistant.	Stain more easily, otherwise the same.
Detection.	Not visible, or visible with difficulty, in living cells unstained.	Not reported in living cells.	Not seen in living cells.
	Demonstrated in teased cells.[23]	Not reported in teased cells.	Not as yet seen in teased cells.
	Occurs in blood in exceedingly small numbers (Ricketts confirmed by Wolbach[23]).	Not as yet clearly seen in blood.*	Observed occasionally within vascular lumen in sections but not in blood smears.
	Detectable in sections after careful search.	More difficult to detect in sections than spotted fever organism.	Easier to observe in sections than either of the others.

Position.	Chiefly within the cytoplasm of endothelial cells, but also in the smooth muscle of the media;[13] in endothelial cells which collect in and around the adventitia;[23] in vascular lumina, giant cells, liver cells, and mononuclear leucocytes.[12]	Restricted to the cytoplasm of endothelial cells.†	Definitely restricted to the cytoplasm of endothelial cells and to portions of them broken off into the vascular lumen. Occasionally the cells rupture and discharge single microorganisms into the blood stream.
Arrangement.	Single and in clumps of varying size.	"Globular massing of organisms is the most characteristic appearance of *Rickettsia* in human lesions."[13]	Spherical clumps are more marked than in either spotted fever or typhus, and attain a much larger size. They are often multiple, several discrete clumps being present within a single endothelial cell.‡
Distribution.	Skin, scrotum, epididymis, testis, thyroid, spleen, lungs, and skeletal muscle[24] and in addition in heart, adrenals, lymph glands, and liver.[12]	Skin in 27 cases, kidneys in 2, femoral vein in 1, testes and adnexa in 5, and brain in 7.[13]	Kidney in 11 cases, cerebral cortex in 7, spleen in 4, lymph glands in 4, cerebellar cortex in 3, suprarenals in 3, corpus luteum in 3, midbrain in 2, medulla oblongata in 2, pancreas in 1, heart muscle in 1, corpus striatum in 1, ovary in 1, salivary glands in 1.

* According to Wolbach, Todd, and Palfrey[13] Ricketts' observation of bipolar microorganisms in the blood of typhus fever patients has not been confirmed.

† On page 189 Wolbach, Todd, and Palfrey[13] state that: "they [*Rickettsiæ*] are found only in the endothelium and never, as in Rocky Mountain spotted fever, in the smooth muscle of the media." On the next page, however, they refer to the finding of *Rickettsiæ* in the mononuclear cells of the perivascular nodules. On page 192 the localization of *Rickettsia* in endothelial cells is again emphasized.

‡ In certain of their morphological features these clumps resemble the so called trachoma bodies described by Noguchi and Cohen (Noguchi, H., and Cohen, M., *J. Exp. Med.*, 1913, xviii, 572, Fig. 1).

TABLE III—*Concluded.*

	Spotted fever.	Typhus fever.	Heartwater.
Association with febrile reaction.	Increase in number progressively with development of the lesions from 1st to 5th or last day of fever when both attain a maximum. Early in reaction diplobacillary becoming bacillary in later stages.[12]	Found in every case (*i.e.* 25) where postmortem examination was made before the 13th day of the disease and while the body was in a fresh condition.[13]	Found in 16 cases, most frequently within the 6 day period after the temperature has commenced to decline.
Association with lesions.	Direct injury caused by parasite shown by degenerative changes in endothelial cells and smooth muscle cells of media.[23]	Similar swelling and degeneration of endothelial cells.	Endothelial cells swollen, but, in contrast, show no sign of degeneration.
	Thrombosis, necrosis, and perivascular infiltration chiefly in subcutaneous tissue associated with the development of a rash.	Similar.	No thrombosis, necrosis, or perivascular infiltration and consequently no rash. The most characteristic lesion is a variable degree of hydropericardium.

"Gram-negative, intracellular, bacterium-like organisms found in arthropods." This inclusion of the word "arthropod" necessitates mention of the fact to be reported in a succeeding paper that similar microorganisms have been found in the insect vector of heartwater[21] so that the *Rickettsiæ* under consideration fulfill this condition also. My discussion will be limited to the microorganisms as seen in mammalian tissues.

In a recent critical review of "the position of *Rickettsia* as an ætiological factor in disease," Arkwright[22] expressed the opinion that *Rickettsiæ* are only known to be concerned with three diseases; namely, Rocky Mountain spotted fever, typhus fever, and trench fever. They have been frequently seen in the invertebrate ectoparasitic hosts in association with all three. In mammalian tissues they have also been reported in Rocky Mountain spotted fever, chiefly by Wolbach[23, 24] and in typhus fever by Kuczinski,[25] by Wolbach and Todd,[18] by Stevenson and Balfour,[26] with certain reservations, and more emphatically by Wolbach, Todd, and Palfrey;[13] but it is questionable whether they have been observed in trench fever.

The kindness of Professor Wolbach and of Dr. Nicholson in giving me slides containing the *Rickettsia* of spotted fever, and of Professor Wolbach in giving me tissues from cases of typhus fever, which I have sectioned and stained myself, makes it possible for me to compare these *Rickettsiæ* very closely with the microorganisms in heartwater. For convenience this comparison is given in tabular form. Rocky Mountain spotted fever is listed first as the only disease in which *Rickettsiæ* have been proved to be the etiological agents and in which they may invariably be detected in the tissues; then typhus fever, in which Wolbach and his colleagues have presented valuable evidence in favor of *Rickettsiæ* as the causative agents; and lastly heartwater.

In addition to this close resemblance between the three microorganisms as brought out in Table III, there are certain points of similarity between the viruses of the three diseases. In each case the virus may be transmitted by the bites of infective insects (or arachnids) or by the inoculation of infective blood in which *Rickettsiæ* are not demonstrable or may be seen with difficulty (Rocky Mountain spotted fever). All three occasion a high temperature reaction and a heavy mor-

---

[21] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 253.

[22] Arkwright, J. A., *J. Roy. Army Med. Corps*, 1924, xlii, 447.

[23] Wolbach, S. B., Rocky Mountain spotted fever, in Bryam and Archibald, The practice of medicine in the tropics, Oxford Medical Publications, Oxford, 1923, iii, 2092.

[24] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 1.

[25] Kuczinski, *Centr. allg. Path. u. path. Anat.*, 1919–20, xxx, quoted from Arkwright, J. A., Typhus fever, in Bryam and Archibald, The practice of medicine in the tropics, Oxford Medical Publications, Oxford, 1923, iii, 2078.

[26] Stevenson, A. C., and Balfour, A., *J. Path. and Bact.*, 1921, xxiv, 289.

tality. The virus of heartwater seems to differ from that of Rocky Mountain spotted fever and to resemble that of typhus fever in respect to the fact that it is not inherited through successive generations in the eggs of the invertebrate vector. All three viruses are unfilterable and as yet uncultivable.[27] They do not retain their vitality even under the most favorable conditions *in vitro* for more than a few days; and one attack of the disease confers a permanent immunity or one lasting for several years.

A divergence in clinical symptoms between heartwater, on the one hand, and spotted fever and typhus fever, on the other, is to be expected, because heartwater is, by contrast, a disease of ruminants only. In all three, however, functional disturbances of the nervous system are of common occurrence. The absence of a rash in heartwater is probably due to the non-involvement of the cutaneous blood vessels. It is this property, that the vascular endothelium in all parts of the body retains its normal vitality and is not injured by the action of the virus, which makes heartwater so very favorable for the study of the general problem of the relation of *Rickettsiæ* to disease. In heartwater the *Rickettsiæ* may be easily studied in endothelial cells which are to all appearances normal except for the mechanical distention which they have undergone to accommodate the *Rickettsiæ* in large numbers. By contrast in both spotted fever and typhus fever the blood vessels are the seat of severe lesions and the endothelium is much involved. This injury results in the appearance of numerous granules within the endothelial cells, which mask the microorganisms. In consequence of this fact investigators have been slow to accept conclusions based wholly upon the histological study of *Rickettsiæ*, particularly so since the *Rickettsiæ* of both spotted fever and typhus have resisted many and repeated attempts at artificial cultivation. Not only is the study of heartwater relieved from this handicap, but in addition the *Rickettsiæ* may be rapidly demonstrated by relatively simple methods of technique.

SUMMARY.

A Gram-negative, intracellular, coccus-like microorganism was found in cases of heartwater in the three species which are susceptible to the disease; namely, goats, sheep, and cattle. It was absent in the case of control animals, both normal ones and those dying of some other diseases. The presence of this microorganism was definitely related to the febrile reaction. It was most easily detected in the renal glomeruli and in the small capillaries of the cerebral cortex but probably occurred throughout the body. The microorganism

[27] In this respect they apparently differ from (1) *Rickettsia melophagi* (Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53); (2) *Rickettsia rocha-limæ* (Weigl, R., *Przeglad Epidemjol.*, 1921, i, 373); and (3) *Rickettsia nipponica* (Sellards, A. W., *Am. J. Trop. Med.*, 1923, iii, 529).

was a typical endothelial parasite, being restricted in distribution to the endothelial cells of the smaller blood vessels and to portions of such elements which had broken off into the blood stream. It was never observed to cause injury to the cells other than those incident to mechanical distention through accumulation within them of many individuals in large densely packed masses which were characteristically spherical. A typical attribute was the presence of several of these masses within the cytoplasm of a single endothelial cell. In view of the association of this microorganism with heartwater, a disease of ruminants, and thus far the only one in which microorganisms resembling *Rickettsiæ* have been reported, the designation *Rickettsia ruminantium* is proposed.

EXPLANATION OF PLATES.

PLATE 10.

The microorganisms in the endothelial lining of small blood vessels are represented as colored with Giemsa's stain. The drawings were made with Zeiss apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida giving a magnification of 1,940 diameters.

FIG. 1. Spleen (G. 4510) fixed in Regaud's fluid. A small branching capillary is shown, the lumen of which is occluded by the enlargement of endothelial cells containing microorganisms.

FIG. 2. Spleen (S. 6821) fixed in Zenker's fluid. A swollen endothelial cell is depicted containing a roughly spherical mass of microorganisms embedded in chromophobic cytoplasm.

FIG. 3. Kidney (G. 4510) fixed in Regaud's fluid. A clump of microorganisms is seen discharging into the blood stream.

FIG. 4. Cerebral cortex (G. 8386) fixed in Zenker's fluid. A clump of microorganisms in the endothelial lining of a small blood vessel.

FIG. 5. Kidney (G. 4510) fixed in Regaud's fluid. An endothelial cell containing two masses of microorganisms with the lumen (at the base of the drawing) almost blocked. In contact with it is an histogenous mast cell possessing granules which are of irregular size and shape and colored deep red.

FIGS. 6 and 7. Cerebral cortex (G. 4510) fixed in Zenker's fluid. Two capillaries with endothelial cells containing clumps of microorganisms surrounded by zones of unstained cytoplasm.

FIG. 8. The same. A rather larger blood vessel with two endothelial cells in different stages of engorgement.

FIG. 9. Kidney (G. 4510). A still larger blood vessel with engorged endothelial cells in which the microorganisms are arranged in multiple sharply outlined clumps which were found to be very characteristic of heartwater.

PLATE 11.

The photomicrographs were taken with Zeiss apochromatic objective 3 mm., 1.40 aperture, and compensating ocular 8 giving a magnification of 1,400 diameters. The preparations were fixed in Zenker's fluid and colored by Giemsa's stain.

FIG. 10. A small blood vessel in the cerebral cortex (G. 4510) in which two clumps of microorganisms are seen within the cytoplasm of endothelial cells. The cell containing the largest clump completely occludes the lumen of the vessel. There is a smaller clump in the next endothelial cell to the left, colored more intensely, since the microorganisms are more closely packed together. Each clump is encircled by a halo of lightly stained cytoplasm.

FIG. 11. A clump of microorganisms within an endothelial cell of a renal glomerulus (G. 8386). In some places it may be seen that the organisms are grouped in pairs and that each is surrounded by a halo.

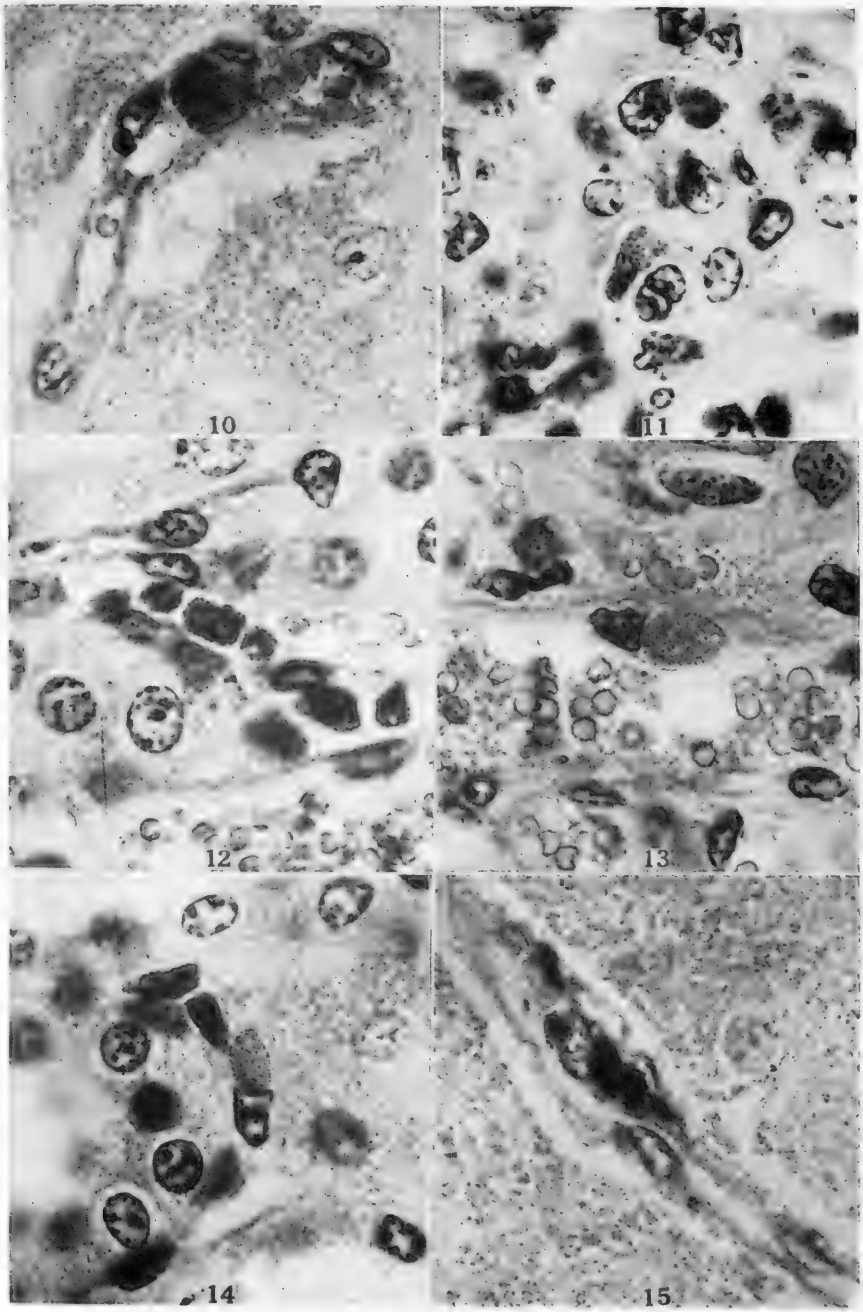
FIG. 12. Four clumps of microorganisms within an endothelial cell of a capillary between the renal tubules (G. 4510). The fourth clump on the left is slightly out of focus. The nucleus is flattened and lies just below the microorganisms.

FIG. 13. A clump of microorganisms in an endothelial cell of a somewhat larger blood vessel of the same kidney.

FIG. 14. Another group of microorganisms within an endothelial cell of a capillary of the same kidney.

FIG. 15. A clump of microorganisms in an endothelial cell of a small blood vessel of the cerebral cortex (G. 8386).

(Cowdry: Etiology of heartwater. I.)



(Cowdry: Etiology of heartwater. I.)

STUDIES ON THE ETIOLOGY OF HEARTWATER.

II. Rickettsia ruminantium (N. Sp.) in the Tissues of Ticks Transmitting the Disease.*

By E. V. COWDRY.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 12 and 13.

(Received for publication, February 25, 1925.)

In the first of these studies[1] attention was directed toward a specific febrile disease of sheep, goats, and cattle in South Africa which is known as heartwater on account of the collection of a variable amount of fluid in the pericardium. Upon histological examination a new kind of *Rickettsia* was found in all three of the species suffering from the disease, which was uniformly absent in controls. The *Rickettsiæ* were present within the endothelial cells of the smaller blood vessels throughout the body but were most easily detected in the brain, kidneys, and spleen. Their occurrence corresponded very closely with the febrile reaction and they disappeared from the tissues soon after the temperature returned to normal in surviving animals. This disappearance coincided with a loss in the infectivity of the blood when it was injected into healthy animals. On the basis of these observations it seemed probable that the *Rickettsiæ* might be the causative agents of heartwater, and since no microorganisms belonging to this general category had hitherto been reported in association with disease in ruminants the term *Rickettsia ruminantium* was adopted for them.

* Second contribution by the South African Expedition of The Rockefeller Institute for Medical Research.

The experiments were made in the Government Laboratories at Onderstepoort and cordial thanks are due to the Government of the Union of South Africa, to Sir Arnold Theiler, and to the members of his staff for the many courtesies extended.

[1] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 231.

The present paper had the object of ascertaining whether these *Rickettsiæ* also occurred in ticks capable of transmitting the disease. For this purpose, and through the foresight of Sir Arnold Theiler, it was possible to experiment with a large number of unfed larvæ all of which were the descendants of a single female belonging to the species *Amblyomma hebræum*, which is known to be capable of transmitting heartwater. The plan adopted was to separate these readily comparable larvæ (numbered 517) into several series some of which were fed on diseased animals and some on normal animals as detailed in Table I. In this way ticks presumably harboring the virus and others known to be free from it, since the virus is not inherited from one generation to another through the eggs,[2] were available for examination and experimentation successively as larvæ, as nymphæ, and as adults.

The methods of tick experimentation and the accurate system of controls devised by Sir Arnold Theiler and by Dr. P. J. du Toit were found to be most helpful. All the ticks were kept in sterilized cotton-plugged test-tubes, and the tubes themselves were placed in glass jars the bottoms of which were covered by thick layers of moistened sand. A large well ventilated room was devoted solely to the care of the ticks. They were protected from direct sunlight and from strong illumination. The history of the larvæ and of the experiments performed with them and their descendants is summarized in Table I which has been extracted from the laboratory records. In this table, passing from left to right, information is given from the origin of the female which laid the eggs, through the subsequent periods of feeding and moulting of the larvæ and nymphæ derived therefrom.

The Infection of Ticks.

Five batches of larvæ, portions of Series 517, were segregated and numbered 604, 605, 606, 607, and 614 respectively (Table I). The first three (Nos. 604, 605, and 606) were fed on normal animals, and the remaining two (Nos. 607 and 614) were fed on animals suffering from heartwater. That the virus was actually in the blood at the

[2] Lounsbury, C. P., *Agric. J. Cape of Good Hope*, 1899; 1900, xvii, 682; 1902, xx, 29; xxi, 22, 165, 221, 315; *Rep. Gov. Entomol.*, 1903, 15; *Agric. J. Cape of Good Hope*, 1904, xiv.

TABLE I.

History of Ticks Employed and Record of Experiments with Them.

Series No.	Origin.	Date.	Eggs laid.	Larvæ hatched.	Larvæ fed on.	Date.	Larvæ dropped off.	Larvæ moulted.	Nymphæ fed on.	Date.	Nymphæ dropped off.	Nymphæ moulted.
		1924	*1924*	*1924*		*1924*	*1924*	*1924*		*1924*	*1924*	*1924*
517	Female from Cow 638.	1/27	2/2	3/10	Bull 424	9/13	9/18					
604	517				Calf 420	8/19	8/25	10/1				
605	517				Sheep 8046	8/19	8/26	10/11				
606	517				" 8046	8/19	8/28, 29, 30	10/11				
607	517				Calf 928	8/27	9/1	10/13				
614	517				Bull 424	9/13	9/18	10/20				
635	614				Sheep 8049	10/22	1/29	12/10				
636	614								Sheep 8049	11/3	11/7	12/10
637	607								Goat 9651	11/3	11/13	12/10
638	614								" 9665	11/3	11/13	12/12
640	614								Goats 8235, 8417	11/22		12/20
647	517				Cow 928		12/17					

time of feeding was proved by inoculation of portions of it into susceptible animals, which, after the usual period of incubation, contracted typical attacks of the disease.

The engorged larvæ of the series fed from the sick animals (Nos. 607 and 614) were allowed to moult, and certain nymphæ which developed from them, renumbered 636, 637, 638, and 640 respectively,

TABLE II.

Examinations.

Series.	Data.	*Rickettsia ruminantium.*	
		Present.	Absent.
Original 517.	Unfed larvæ 189 days after hatching.	0	50
Control 604	No. of days after commencing to feed as larvæ: 51, 53, 57–59, 61–64, 75–79, 84, 85, 96, 105, 112.	0	60
" 605	Same: 75, 104, 106, 111.	0	35
" 606	" 67, 75, 104, 111.	0	47
Infective 607	" 30, 58–60, 61, 62, 64, 67, 68, 70, 71, 81, 84, 89, 101.	35	59
" 614	Same: 13, 14, 16, 20, 22, 23, 27, 28–34, 40, 41, 45, 46, 47, 50, 54, 55, 57, 68.	20	102
Control 635, originally 517.	Same: 47.	0	1
Infective 636, originally 614.	" 75, 82, 96 (5 nymphæ and 4 adults).	4	5
Infective 637, originally 607.	Same: 96 (nymphæ).	1	2
Infective 638, originally 614.	" 96 (2 " and 2 adults).	3	1
Control 647, originally 517.	No. of days after ceasing to feed as larvæ: 5.	0	2

were fed upon normal animals. Some of these latter developed heartwater, as will be related subsequently (see page 266). It was felt that decisions as to the presence or absence of *Rickettsiæ* could only be based upon very thorough study of the ticks. For this reason, although comparatively few specimens were examined, it is probable that the results are more dependable than if many hundreds had been superficially observed. The histological examinations made are listed in

Table II. In the first column, the number and character of the series is given; in the second, the time the examinations were made after the ticks were fed as larvæ (the days when *Rickettsiæ* were observed being underlined); and in the third, the findings in regard to the presence or absence of *Rickettsiæ*.

Method of Examining the Ticks.

As a routine procedure and in the majority of cases the ticks were studied in sections, but this method was often supplemented by smears and by teasing out the living cells in sterile salt solution and observing them by oblique illumination and by direct illumination, unstained and with the aid of various vital dyes. Special treatment was naturally required in each stage of the life cycle.

Unfed larvæ soon after they hatched from the eggs (Series 517) were of such small size that large numbers (twenty-five or more) were fixed (in Regaud's fluid) at one time. They were allowed to settle in a fairly compact mass in the paraffin before solidifying. The clumps of larvæ thus formed cut with difficulty, but a few good sections were often obtained. In making smears ten or more larvæ were applied successively to a single slide. Care was taken to smear them roughly parallel so that each specimen could be followed from end to end with the aid of a mechanical stage.

Engorged larvæ were also found exceedingly difficult to properly fix and section on account of the large amount of blood contained in their intestines, their very resistant integument, and their relatively small size. Accordingly, chief reliance was placed on smears and on the examination of living tissues. The smears were made, as in the case of the unfed larvæ, with the help of sterile instruments upon clean slides without the addition of any saline solution. By this method drying was almost instantaneous. Before staining, the smears were fixed in absolute alcohol, which gave better results than methyl alcohol.

The nymphæ, being slightly larger and less brittle, were cut into sections comparatively easily. In order to prevent the chitin from hardening, the time required for dehydration and for embedding was reduced to a minimum. The penetration of the fixative was facilitated by the removal of the legs and by making a small incision in the integument. The sections were cut at a thickness of 4 or 5 μ, but even with the greatest care a complete series of sections was never obtained. At first Zenker's fluid was employed as a preservative, but this was later abandoned on account of a crystalline deposit produced by it, for the most part within the intestinal lumen. This deposit was not removed by the action of Lugol's iodine solution followed by a 5 per cent solution of sodium hyposulfite, but it was dissolved by allowing the sections to remain for a few minutes in a 1 per cent solution of potassium permanganate followed by rinsing them in 5 per cent oxalic acid. This treatment, however, partially destroyed the contrast

coloration with Giemsa's stain. For this reason Regaud's mixture was later used, consisting of 4 parts of a 3 per cent solution of potassium bichromate and 1 part of commercial formalin.[3] The intensity of the subsequent coloration by Giemsa's stain was increased by the mordanting action of the formalin so that it was necessary to render the staining fluid feebly alkaline and to add methyl alcohol as advised by Wolbach.[4] Other fixatives and stains were employed for special purposes (see page 260), and the tissues were examined in smears and in the living state.

In the case of *adults* it was necessary to remove all traces of the chitinous exoskeleton in order to secure good sections. It was found that this was easily accomplished after a few hours preliminary fixation in formalin or Regaud's fluid which made the tissues firmer and greatly facilitated the stripping off of the chitin. Slightly better results were obtained when the ticks were partially dehydrated in the alcohols before commencing to remove the chitin, because this treatment made the viscera more cohesive. Another device was used with engorged nymphs as well as with adults. This consisted of squeezing out the viscera through a small incision in the exoskeleton. A small pair of artery clamps proved much more helpful than ordinary forceps because with them the pressure could be regulated and maintained. After a little practice and with the use of a clean wet needle the viscera could, in most instances, be freed as a single drop, which kept its shape when it fell into the fixative and was consequently very easily imbedded and sectioned. For this purpose Regaud's fluid gave poor results as compared with Zenker's fluid, Giemsa's sublimate, and sublimate acetic, because in it the tissue tended to disperse instead of retaining its spherical shape.

Microorganisms Found.

From the outset a relatively large, Gram-negative, very pleomorphic, typically intracellular, bacterium-like microorganism was invariably found to be present within the lining epithelial cells of the Malpighian tubules of all ticks (larvæ, nymphæ, and adults), both infective and non-infective. This was the same microorganism as that previously reported in the eggs of the specimens of *Amblyomma hebræum* sent by Sir Arnold Theiler to The Rockefeller Institute.[5] Since it was shown that it occurs likewise in unfed larvæ (Series 517)

[3] A modified Regaud's fluid is recommended for the study of *Rickettsiæ* in lice (Rosenberger, G., *Arch. Schiffs- u. Tropen-Hyg.*, 1922, xxvi, 112).

[4] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 1.

[5] Cowdry, E. V., *J. Exp. Med.*, 1923, xxxvii, 431.

which had been reared in sterile test-tubes and had taken no food, it was concluded that it constitutes an hereditary infestation.[6]

Rickettsia ruminantium, on the other hand, was only observed in infective ticks of Series 607 and 614. Because the life histories both of it and of the microorganism above referred to are imperfectly known, there is always the possibility that in some stages the two may be confused. This is particularly to be guarded against in the study of

TABLE III.

Comparison of the Pleomorphic Microorganism and Rickettsia ruminantium.

	Pleomorphic microorganism.	*Rickettsia ruminantium.*
Morphology.	Rods and filaments 0.5–1.5 by 1–5 μ; very pleomorphic.	Cocci and diplococci; pairs 0.25–0.3 by 0.5–0.8 μ; very regular.
Staining reactions.	Gram-negative. Usually red with Giemsa; after formalin fixation stains lightly by Goodpasture's fuchsin method;* fragile, not easily fixed; stains heterogeneously, revealing chromophilic areas.	Gram-negative; usually blue with Giemsa; after formalin fixation stains electively and intensely by Goodpasture's fuchsin method; not fragile, very easily fixed. Stains homogeneously, revealing no internal structure.
Position.	Intracellular in egg cells and Malpighian tubules.	Intracellular and sometimes extracellular in alimentary tract; not in egg cells or Malpighian tubules.
Occurrence.	In all larvæ, nymphæ, and adults infective and non-infective; inherited through the eggs.	Only in infective specimens, and not inherited through the eggs.

* Recommended for *Rickettsiæ* by Hertig and Wolbach (Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1923–24, xliv, 329).

the multiplicative phases in smears when the two microorganisms are nearly of the same size and their relations to the cells of the tick have been lost. They have been contrasted in Fig. 7. The large pleomorphic microorganisms are seen in the cells of the Malpighian tubule above and small clumps of the much more minute *Rickettsia ruminantium* are represented in contiguous intestinal epithelial

[6] It is proposed to discuss this microorganism together with others occurring in ticks and transmitted hereditarily in a subsequent paper (Cowdry, E. V., *J. Exp. Med.*, 1925, xli, 817).

cells below. The first are colored red by Giemsa's stain, and the second blue. Other criteria of distinction are listed in Table III.

Specimens of *Amblyomma hebræum* of unknown history were found to be occasionally infested with a third type of microorganism[5]—a Gram-positive bacillus, approximately 0.25 by 1 μ, parasitic in the cells of the salivary glands; but this microorganism was not observed in the carefully protected ticks under experimentation. It is only mentioned here so that subsequent investigators may at once eliminate it in any consideration of the etiology of heartwater.

Distinctive Characters of Rickettsia ruminantium as Observed in Infective Ticks.

Attempts to examine *Rickettsia ruminantium* in the living state within intestinal epithelial cells teased out in sterile saline solution and in the contents of the gut yielded only indecisive results owing to uncertainty in identification. The main difficulty was to distinguish between the *Rickettsiæ* and some granules from the salivary glands of about the same size which tended also to occur in similar spherical clumps. But the differentiation was clearly made upon the addition of a little of the relatively pure medicinal methylene blue of Meister, Lucius, and Bruening (prewar product). In a concentration of about 1:20,000 this dye colored both the granules and the *Rickettsiæ*, the latter rather more intensely. The *Rickettsiæ* were less refractile and did not possess the definite contours of the granules. They were not motile. Each blue-colored *Rickettsia* seemed to be surrounded by a very thin layer of unstained substance which created the illusory impression that the *Rickettsiæ* were less closely packed together than the granules. When brilliant cresyl blue (Grübler) was applied in place of methylene blue, the *Rickettsiæ* became stained light purple, but were not so clearly seen. The *Rickettsiæ* were also colored by neutral red (Leopold Casella and Co.), but it was more difficult to detect them owing to the strong affinity of many other granules for this dye.

The examination of living cells and of the contents of the gut by dark-field illumination, even under the most favorable optical conditions, proved unprofitable because myriads of mitochondria of approximately the same size and shape as the *Rickettsiæ* were revealed so clearly that any attempt at differentiation between the two would have been both arbitrary and futile. This confusing element could

perhaps have been predicted on the basis of observations recorded in the literature relative to the mitochondria in *Ixodes reduvius*,[7] *Argas mineatus*,[8] and *Amblyomma americana*.[5] The distinction between mitochondria and *Rickettsiæ* was made beyond peradventure in fixed tissues as will be related in the following paragraphs.

Much information was gained by the study of air-dried smears fixed in absolute alcohol and colored by Giemsa's method. But in the examination of the smears it was necessary again to carefully distinguish between the *Rickettsiæ* and these granules of the salivary glands. Like the *Rickettsiæ* these granules often occurred in spherical clumps and were of very minute and fairly uniform size, but they always stained (by Giemsa) a light brick-red color and differed from the *Rickettsiæ* in the finer points of their morphology. By contrast the clumps of *Rickettsiæ* were usually colored blue or light purple (Fig. 3) and in places where the individual microorganisms had been separated it was observed that many of the *Rickettsiæ* were composed of two materials, one colored red and the other very pale blue, as illustrated in Fig. 1. The red-staining material was present in two minute spherules embedded in the blue-staining substance which intervened between them. The ends of the pairs, composed of the red-staining spherules, were uniformly rounded. The intervening blue-staining substance frequently possessed a greater diameter than the spherules. The distance between the spherules was greater than that generally seen in the case of pairs of diplococci. In some cases this distance varied somewhat, suggesting degrees of separation after transverse division. These properties called at once to mind the similar appearance in smears of the *Rickettsiæ* of typhus and of Rocky Mountain spotted fever and of *Rickettsia nipponica* as cultivated by Sellards.[9] But a very exhaustive search failed to reveal any filamentous forms in the case of *Rickettsia ruminantium*.

Rickettsia ruminantium was most profitably studied in sections and its relation to the intestinal epithelial cells determined. Its most characteristic feature, already alluded to, was its habit of occurring in densely packed spherical clumps, precisely as in the endothelial cells

[7] Nordenskiöld, E., *Zool. Anz.*, 1909, xxxiv, 511.
[8] Casteel, D. B., *J. Morphol.*, 1916–17, xxviii, 643.
[9] Sellards, A. W., *Am. J. Trop. Med.*, 1923, iii, 529.

of animals suffering from heartwater. This clumping is well illustrated in Figs. 2 to 7, which should be compared with Figs. 1 to 9 in the first part of this report dealing with the same *Rickettsia* in mammalian tissues.[1] The clumps varied greatly in size and several of them were often found within the cytoplasm of a single cell (Figs. 5 to 7), just as in mammals. The tendency noted in mammals for the masses of *Rickettsiæ* to be surrounded by a kind of halo of clear cytoplasm, was less marked in the ticks, probably because the cytoplasm of the intestinal epithelial cells was very fluid and contained comparatively little stainable ground substance, or perhaps on account of the absence of intracellular digestion of the *Rickettsiæ* in them. In some cases in the ticks it was seen that these clumps were distinctly heterogeneous in character, being composed of the *Rickettsiæ* themselves, and of a matrix, which differed in staining properties from the ground substance of the cells. This matrix was detected in specimens of Series 607 fixed in both Regaud's fluid and 10 per cent formalin and colored by Giemsa's stain which was only slightly differentiated. It is illustrated in light blue in Fig. 4, *d* and *e*. Its occurrence, however, was not constant even in neighboring clumps of *Rickettsiæ* in the same section which were presumably colored in exactly the same way. The matrix seemed to be most frequently met with in the case of masses of *Rickettsiæ* which were not very tightly packed together. Experimental evidence that the individual *Rickettsiæ* composing a clump were very cohesive was obtained in the examination of living tissues and of smears in which the clumps often remained intact (as represented in Fig. 3), although they had been subjected to considerable mechanical traction. The clumps increased progressively in size and in number during the first few days after their first appearance, and in the older nymphæ the *Rickettsiæ* were observed to escape into the lumen of the intestine. In this new location also they usually retained their clump-like association (in spite of the fact that they were suspended in a more fluid medium), but they were occasionally scattered about singly and in small irregular groups.

When closely crowded together the *Rickettsiæ* had the appearance of minute cocci about 0.2 to 0.3 μ in diameter as illustrated in Figs. 8 to 13. When, however, the plane of section passed barely through the edge of a mass of *Rickettsiæ*, the optical effect of superposition was

avoided and it was observed that some of them were bacillary in shape and others diplococcal. The bacilli measured about 0.2 to 0.3 by 0.4 to 0.5 μ, as seen after fixation in Regaud's fluid and coloration by Giemsa's stain. The pairs of diplococci were approximately 0.2 by 0.8 μ. It is important to note that the coccal forms were sometimes swollen by the action of formalin as indicated in Fig. 4, *f* and *g*.

Certain atypical bodies possibly, but not probably, related to the *Rickettsiæ* made their appearance in sections of ticks of Series 607, examined as nymphæ, 78 days after they commenced to feed on an infected animal. They were larger than most *Rickettsiæ*, of bacillary or coccoid shape, sometimes disposed in pairs, and usually embedded in the substance of clumps of true *Rickettsiæ*. After Regaud's fixation they were colored dark red by Giemsa's stain, instead of some shade of blue or purple. These atypical bodies are illustrated in Figs. 4, *a*, *b*, and *c* and 10. They were not seen in older nymphæ which had become engorged with blood by feeding on animals to which they transmitted heartwater, although the regular forms of *Rickettsiæ* were abundant in the lining epithelial cells of the intestine and free in the lumen. Nor were they observed in adults.

The duality in staining property, observed in the smears, was not clearly seen in sections—an experience also reported by Hertig and Wolbach[10] in their study of *Rickettsiæ* in arthropods. In sections the *Rickettsiæ* of heartwater were usually stained only one color, with the possible exception of the questionable atypical forms already mentioned. The reactions of the *Rickettsiæ* were, as was to be expected, more dependent upon the fixative (and its mordanting action) than upon the stain. For example, with Giemsa's stain they were colored clear blue, after Zenker's fluid with or without acetic acid; bluish purple, after Regaud's fluid, formalin, or Carnoy's fluid; pale green, after Flemming's fluid; and light pink after the picrosulfuric mixture of Mayer.[11] The *Rickettsiæ* were Gram-negative and stained well by Goodpasture's fuchsin method and by Unna's alkaline methylene blue after appropriate preservations. In stained preparations their outlines were somewhat less distinct than those of most bacteria, a phenomenon which da Rocha-Lima[12] noted in his description of the type species, *Rickettsia prowazeki*.

[10] Hertig, M., and Wolbach, S. B., *J. Med. Research*, 1923–24, xliv, 329.
[11] Mayer, *Mitt. zool. Station Neapel*, 1880, ii, 2.
[12] da Rocha-Lima, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

As observed in sections, the intestinal epithelial cells containing the *Rickettsia* showed no recognizable signs of injury, as was the case also with the *Rickettsia*-laden endothelial cells of mammals suffering from heartwater. This is an important point of similarity with the *Rickettsia* of Rocky Mountain spotted fever as seen in the tissues of the tick *Dermacentor venustus* and with many other *Rickettsiæ* which typically establish relations with their arachnid hosts approaching true symbiosis.

In describing the *Rickettsiæ* just mentioned, as they occur in mammalian tissues it was shown[1] that they are true microorganisms which need not be confused with mitochondria, or with any other normal cellular component, or with any product of cellular degeneration or of injury. Much of the evidence then advanced is applicable also to these *Rickettsiæ* in ticks. It will suffice to recall their characteristic morphology and staining properties, their presence in infective ticks, and their absence in controls.

Relation of Rickettsia ruminantium to Infectivity in Ticks.

Rickettsia ruminantium was observed in the two strains of ticks (*i.e.* Nos. 607 and 614) which had fed upon cases of heartwater and was uniformly absent in the three series of controls (*i.e.* Nos. 604, 605, and 606).

In Series 607 it was first seen in nymphæ developed from larvæ which had fed upon a case of heartwater 61 days previously. But in Series 614 it was detected considerably earlier, in one larva 27 days after feeding. It was not, however, again seen in this series (No. 614) until 40 days after feeding, when the larvæ had moulted.

Repeated attempts to bring the *Rickettsia* to light in both series in this interval were unsuccessful. Nor could the infectivity of the larvæ at this time be tested experimentally because they were engorged with blood and consequently were not in a condition to feed. It is possible that during this period *Rickettsiæ* were present singly or in relatively small aggregates and were rapidly bleached in the differentiation of the stain. The corresponding failure[1] to observe them in the circulating blood of mammals, the infectivity of which was easily proved by subinoculation, may be susceptible of a similar explanation.

After the 61st day in Series 607, and the 40th day in Series 614, the

Rickettsiæ were regularly found in about 75 per cent of the nymphæ examined. It is probable that in Series 607 they occurred in small masses before moulting, as in Series 614, but escaped recognition.

TABLE IV.

Summary of Experiments Showing the Infectivity of Ticks Containing Rickettsia ruminantium.

Ticks of series.	Fed on.	Consequences.
614 renumbered 636.	Sheep 8049	After an incubation period of 16 days, there occurred an abrupt rise of temperature to 106.8°, which was maintained at about 105.8° for 4 days, then fell to normal and the animal recovered.
607 renumbered 637.	Goat 9651	No reaction during 19 days of observation. (This animal was probably immune.)
614 renumbered 638.	" 9665	After an incubation period of 14 days temperature rose suddenly to 107°, when the animal was killed.
614 renumbered 640.	Goats 8235, 8417	In Goat 8235 after an incubation period of 13 days temperature rose to 106° and oscillated for 4 days between 106° and 104°, when the animal died. In Goat 8417 after an incubation period of 18 days there was a sudden rise in temperature to 106° which was maintained for 3 days, when it fell to normal and rose again to about 106° on the 25th day. Again the fever was maintained for 4 days, when it subsided and the animal was discharged.
614 The engorged nymphæ were not renumbered.	Goat 9662	After an incubation period of 12 days, an abrupt rise in temperature to 107.0° occurred, which was maintained with slight oscillations for 7 days. Then it commenced to fall and the animal died.

Contrary to expectations, the *Rickettsiæ* seemed to be strictly limited to the epithelial cells of the intestine and to the lumen of the gut. They were never detected with certainty in the cells of the sali-

vary glands. Perhaps in this location also they may have escaped observation because the accurate identification of a single *Rickettsia* embedded in cells which are so highly granular, even under the most favorable conditions, would naturally be a problem of considerable difficulty, particularly if the *Rickettsia* happened to be of spherical shape. But although infection may take place through the pouring of salivary secretion, containing *Rickettsiæ*, into the wound at the time of feeding, this is not necessarily the case.

An example of another method of transmission is afforded by the tick *Ornithodorus moubata*. It will be recalled that Leishman has brought forward considerable evidence to show that at ordinary temperatures the salivary glands of *Ornithodorus moubata* do not harbor *Spirochæta duttoni* and that infection with relapsing fever probably takes place either by regurgitation, or by the excretion, of infective matter from the alimentary canal. In either case the spirochetes would penetrate through the wound caused by the tick's bite.[13]

The experiments made in the tick transmission of the disease have been summarized in Table IV.

In brief, feeding the nymphæ which possessed *Rickettsiæ* in their alimentary tracts on susceptible sheep and goats resulted in typical attacks of heartwater, and in the tissues of the sick animals identical *Rickettsiæ* made their appearance. Thus, in ticks, as in mammals the *Rickettsiæ* proved to be inseparable from the heartwater virus. Like the virus, they were acquired when larvæ were fed on animals suffering from heartwater and containing *Rickettsiæ*. Like the virus, also, they passed through the first moulting period into nymphs in which their presence coincided with the presence of the virus, or, in other words, with the ability to transmit heartwater. It is known that the virus will pass the second moulting period into adults and in these also I have found the *Rickettsiæ* (Table II, Series 638; Fig. 13).

That *Rickettsia ruminantium* is a highly specific microorganism and is confined to the heartwater tick, *Amblyomma hebræum*, was determined by an examination of preparations made primarily for other purposes[5,6] of several other ticks the names of which follow: *Amblyomma americana, Amblyomma maculatum, Amblyomma tuberculatum, Argas persicus, Boophilus decoloratus, Dermacentor albipictus, Dermacentor*

[13] Quoted from Hindle, E., *Parasitology*, 1911, iv, 133.

variabilis, *Dermacentor venustus*, *Hæmophysalis leachi*, *Hæmophysalis leporis palustris*, *Hyalomma ægyptium*, *Margaropus annulatus*, *Margaropus annulatus australis*, *Margaropus winthemi*, *Ornithodorus moubata*, *Ornithodorus megnini*, *Ornithodorus turicata*, *Rhipicephalus appendiculatus*, *Rhipicephalus capensis*, *Rhipicephalus evertsi*, *Rhipicephalus pulchellus*, *Rhipicephalus sanguineus*, and *Rhipicephalus simus*.

DISCUSSION.

In concluding the first section of this paper on heartwater,[1] *Rickettsia ruminantium*, as it appeared in the tissues of animals suffering from the disease, was compared with the *Rickettsiæ* which are associated with Rocky Mountain spotted fever and with typhus fever as observed in the tissues of man and other mammals.

In order to supplement this comparison and to furnish more complete data Table V was prepared giving the properties of *Rickettsiæ* as seen in the arthropod vectors.

For convenience the comparison has been made along the same lines as in Paper I, of which it is merely a continuation. The chief difference is that in this case trench fever has also been considered, since the *Rickettsiæ* presumably concerned with it are well known in lice. The comparison of *Rickettsia ruminantium* with the *Dermacentroxenus rickettsia* of spotted fever has been greatly facilitated by the kindness of Dr. R. R. Parker of Hamilton, Montana, in sending to Dr. Noguchi an abundant supply of ticks, infected with the virus of spotted fever, some of which Dr. Noguchi very courteously made available for histological study.

This table shows that *Rickettsia ruminantium*, as it exists in its arthropod host, resembles the type species, *Rickettsia prowazeki*, rather more closely than does the *Rickettsia* of Rocky Mountain spotted fever, particularly in its position within the lining epithelial cells of the alimentary tract and in the absence of intranuclear forms. Filamentous forms were not observed, as in the other three *Rickettsiæ*, but, like them, *Rickettsia ruminantium* was Gram-negative, showed red- and blue-staining materials on division, possessed rather less sharply outlined contours than most bacteria, and was of very minute size.

Concerning the causative relationship of *Rickettsiæ* to these three

TABLE V.

Comparison of the Rickettsiæ of Rocky Mountain Spotted Fever, Typhus Fever, Trench Fever, and Heartwater as Seen in the Arthropod Vectors.

	Spotted fever (*Dermacentroxenus rickettsia*).	Typhus fever (*Rickettsia prowazeki*).	Trench fever (*Rickettsia quintana*).	Heartwater (*Rickettsia ruminantium*).
Morphology.	More pleomorphic than in infected mammals. (*a*) Extranuclear bacillus-like form without chromatoid granules 0.5 to 1 μ in length. (*b*) Very minute rods with chromatoid granules within nuclei. (*c*) Larger lanceolate paired forms.* "Less bacterium-like than any of the *Rickettsiæ*."† "Does not occur in thread-like or filamentous forms as does *Rickettsia prowazeki*."†	More pleomorphic than in infected mammals. 0.3 by 0.4 μ for single elements and 0.3 by 0.9 μ for double.[12]	Pleomorphic, not definitely known in man.‡ 0.3 by 0.3 or 0.3 by 0.5 μ; round, oval, diplococci or bacillary with stained poles.§ "More uniform in morphology, plumper and more definitely oval than *Rickettsia prowazeki*."† "Shorter and thicker than the bacilli of typhus."§	More pleomorphic than in infected mammals. Cocci 0.2 to 0.3 μ in diameter; bacilli 0.2 to 0.3 by 0.4 to 0.5 μ; and diplococci 0.2 by 0.8 μ; no filamentous forms observed.
Phases of multiplication.	Intracellular and intranuclear multiplication. "In multiplicative form always shows red- and blue-staining materials."†	"Multiplies exclusively within cells in the louse;" also shows† red- and blue-staining materials.	Multiplies within the lumen of the intestine of the louse and on the surface of the epithelial cells.	In multiplicative form also shows red- and blue-staining materials; multiplication takes place within the intestinal epithelial cells and perhaps in the intestinal lumen.

Microchemical reactions.	Gram-negative extranuclear bacillary forms stain pale blue with Giemsa; minute rods stain blue with deeply colored chromatoid granules; and larger lanceolate paired forms, purple.* Outlines less sharp than those of most bacteria; not acid-fast.	Gram-negative; stains feebly by basic aniline dyes, pale blue by Giemsa;† outlines less sharp than those of most bacteria; not acid-fast.	Gram-negative; "easier to stain than *Rickettsia prowazeki*."† Purple or red by Giemsa;† outlines less sharp than those of most bacteria; not acid-fast.	Gram-negative; easily stained dark blue by Giemsa; outlines less sharp than those of most bacteria; not acid-fast.
Detection.	Very simple in well fixed tissues and smears and much easier than in infected mammals.	The same.	The same.	The same.
Motility.	Absent.	Absent.	Absent.	Absent.
Position.	Extracellular, intracellular, intranuclear.	Extracellular, intracellular; never intranuclear.	Chiefly extracellular;‖ never intranuclear.	Sometimes, extracellular, but chiefly intracellular; never intranuclear.

* Wolbach, S. B., Section on Rocky Mountain spotted fever, in Bryam and Archibald, The practice of medicine in the tropics, Oxford Medical Publications, Oxford, 1924, iii, 2100.

† Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

‡ The observation of diplobacillary and diplococcoid bodies in the blood of patients suffering from trench fever by Töpfer (1916) and by Jungmann and Kuczynski (1917) is not accepted by Munk and da Rocha-Lima (Munk, F., and da Rocha-Lima, H., *Münch. med. Woch.*, 1917, xliv, 1422) and subsequent workers, although there is some collateral evidence indicating the probability of their occurrence.

§ Töpfer, H., *Münch. med. Woch.*, 1916, xliii, 1495.

‖ Although "Sikora and others maintain that *Rickettsia pediculi* invades the cells of the louse's stomach" (quoted from Wolbach, Todd, and Palfrey,† who take the position that *Rickettsia pediculi*, *Rickettsia quintana*, and *Rickettsia wolhynica* are morphologically indistinguishable and in all probability identical).

TABLE V—*Concluded.*

	Spotted fever (*Dermacentroxenus rickettsia.*)	Typhus fever (*Rickettsia prowazeki*).	Trench fever (*Rickettsia quintana*).	Heartwater (*Rickettsia ruminantium*).
Arrangement.	Haphazard: singly, end to end, or in clumps, depending upon physical conditions in the tissues.	Haphazard, in very large masses.	In layers upon surface of intestinal epithelium.	Densely packed together in very characteristic clumps which are usually spherical and several of which may occur in the cytoplasm of a single cell.**
Distribution.	In all the tissues of the tick in certain phases of the life cycle, at other times more restricted.	In the epithelial cells lining the alimentary tract and in the lumen.	Within lumen of alimentary tract.	In epithelial cells lining alimentary tract and in lumen.
Effect on host.	Not harmful to ticks.	"Eventually causes the death of the louse through suspension of digestion."†	Not harmful to lice.	Not harmful to ticks.
Infestation.	Hereditary.	Not hereditary.†	Not hereditary.†† Hereditarily transmitted.†	Not hereditary.

** In this respect these *Rickettsiæ* exhibit a strong morphological resemblance to the trachoma bodies described by Noguchi and Cohen (Noguchi, H., and Cohen, M., *J. Exp. Med.*, 1913, xviii, 572, Fig. 1).

†† Arkwright, J. A., Bacot, A., and Duncan, F. M., *J. Hyg.*, 1919–20, xviii, 76.

diseases and to heartwater, there remains, however, an element of uncertainty, because in none of them have the *Rickettsiæ* been isolated in pure cultures and the disease produced by inoculation therewith. In the case of tsutsugamushi disease, conditions seem to be reversed, inasmuch as Sellards[9] has apparently been successful in cultivating a *Rickettsia*-like microorganism which has not thus far been clearly observed in the tissues of patients suffering from the disease or in the arachnids which transmit it. His observations furnish a point of departure for the histological examination of the tissues in tsutsugamushi disease and may point the way to the successful cultivation of other pathogenic *Rickettsiæ*. In the earlier studies on *Rickettsiæ* their failure to grow upon artificial media was frequently cited as a point of importance in identification. Happily this difficulty is in process of being overcome in *Rickettsia melophagi*[11] and *Rickettsia rocha-limæ*,[15] which most closely approximate to ordinary bacteria in their properties. One of the least bacterial in nature, *Dermacentroxenus rickettsia*, has been successfully propagated in tissue cultures by Wolbach and Schlesinger[16] and *Rickettsia ctenocephali* has been cultivated in the celom of the body louse by Sikora.[17]

In a recent paper Arkwright[18] expressed the opinion that *Rickettsiæ* are only known to be concerned with three diseases, Rocky Mountain spotted fever, typhus fever, and trench fever. To these three we may add heartwater, and possibly, as a result of Sellards' work, tsutsugamushi disease also. If the observations reported in the present paper are confirmed, the recognition of typical *Rickettsiæ* in heartwater will enlarge our conception of the scope of the pathogenicity of these microorganisms, which has hitherto been restricted to man and to experimentally infected animals, by the inclusion of a disease of domestic animals which is never found in man. The bringing of new diseases under the general heading of "Rickettsiosis" suggests the likelihood that the limitations of this group have not yet

[14] Nöller, W., *Arch. Schiffs- u. Tropen-Hyg.*, 1917, xxi, 53.
[15] Weigl, R., *Przeglad Epidemjol.*, 1921, i, 373.
[16] Wolbach, S. B., and Schlesinger, M. J., *J. Med. Research*, 1923–24, xliv, 231.
[17] Sikora, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1921, xxv, 123.
[18] Arkwright, J. A., *J. Roy. Army Med. Corps*, 1924, xlii, 447.

been established and that other febrile diseases of unknown etiology, which are insect-transmitted, may eventually be found to fall in the same category.

SUMMARY.

The evidence offered in the first of these studies[1] indicative of a causative relationship between *Rickettsia ruminantium* and heartwater is supplemented by the following observations concerning the ticks which carry the disease.

When larvæ, which had taken no food since hatching, were allowed to feed upon cases of heartwater, they acquired *Rickettsiæ* which appeared to be identical with those in the tissues of animals suffering from heartwater, whereas control larvæ hatched from eggs deposited by the same female and fed on normal animals remained free of *Rickettsiæ*.

After larvæ presumably infective had moulted, the resultant nymphæ containing *Rickettsiæ* in their alimentary tracts, when fed upon susceptible animals produced in them typical attacks of heartwater, which the control nymphæ, devoid of *Rickettsiæ*, failed to do. The tissues of animals thus infected were found upon histological examination to contain typical *Rickettsiæ*.

EXPLANATION OF PLATES.

PLATE 12.

The figures were drawn with Zeiss apochromatic objective 1.5 mm., compensating ocular 8, and camera lucida, and have been reproduced without reduction so that they represent a magnification of 1,940 diameters.

FIG. 1. *Rickettsiæ* composed of two materials, one colored faint red or purple and the other light blue. From a smear of a nymph of Series 614, made 63 days after the nymph had commenced to feed as a larva on Bull 424 suffering from heartwater. The smear was dried in air, fixed in absolute alcohol, and colored by Giemsa's stain.

FIG. 2. Large clump of *Rickettsiæ* from a section of a nymph of Series 607 made 63 days after feeding on Calf 928 suffering from heartwater. Fixed in Regaud's fluid and colored by Giemsa's stain.

FIG. 3. Clumps of *Rickettsiæ* in a smear of a nymph of Series 614 made 58 days after feeding on Bull 424. Fixed in absolute alcohol and colored by Giemsa's stain.

FIG. 4, *a* to *g*. (*a*), and (*b*), (*c*) Three clumps of *Rickettsiæ* in intestinal epithelial

cells showing certain larger more irregular bodies stained dark red. From a section of a nymph of Infective Series 607 fixed, 77 days after feeding, in Regaud's fluid and colored by Giemsa's stain. (*d*) A single clump of *Rickettsiæ* embedded in a blue-stained matrix. From a section of a nymph of Infective Series 607 fixed, 79 days after feeding, in 10 per cent formalin and colored by Giemsa's stain. (*e*) Another clump of more lightly colored *Rickettsiæ* within a blue-stained matrix. From a section of a nymph of Infective Series 607, fixed, 88 days after feeding, in Regaud's fluid and colored by Giemsa's stain. (*f*) and (*g*) Two clumps of *Rickettsiæ* swollen by the action of formalin. From the same preparation as (*d*).

FIG. 5. Two clumps of *Rickettsiæ* within an intestinal epithelial cell. From a nymph of Infective Series 607, fixed in Regaud's fluid 69 days after feeding, and colored by Giemsa's stain.

FIG. 6. Large intestinal epithelial cell containing several clumps of *Rickettsiæ* from a nymph of Infective Series 607, fixed in Regaud's fluid 64 days after feeding and colored by Giemsa's stain.

FIG. 7. A portion of the wall of a Malpighian tubule, and, below, adjacent epithelial cells of the intestine. The cells of the Malpighian tubule contain large bacillary microorganisms and the intestinal cells clumps of *Rickettsiæ*. Neither of the two types of cells shows signs of injury. From a nymph of Infective Series 607 in Regaud's fluid 70 days after feeding and colored by Giemsa's stain.

PLATE 13.

The photomicrographs were taken with Zeiss apochromatic objective 3 mm., 1.40 aperture, and compensating ocular 8, giving a magnification of 1,400 diameters.

FIG. 8. Two clumps of *Rickettsiæ*, one spherical, very densely packed, and to the left, the other slightly to the right of the center and showing well the individual *Rickettsiæ*. From a nymph of Infective Series 607, taken 84 days after feeding as a larva, fixed in 10 per cent formalin, and stained by Goodpasture's fuchsin method.

FIG. 9. Intestinal epithelial cells containing one very large and deeply stained oval clump of *Rickettsiæ* to the left, a somewhat smaller one to the right, and still smaller, scattered, irregular masses of *Rickettsiæ*. From a nymph of Infective Series 614, 67 days after feeding, fixed in Regaud's fluid and colored by Giemsa's stain.

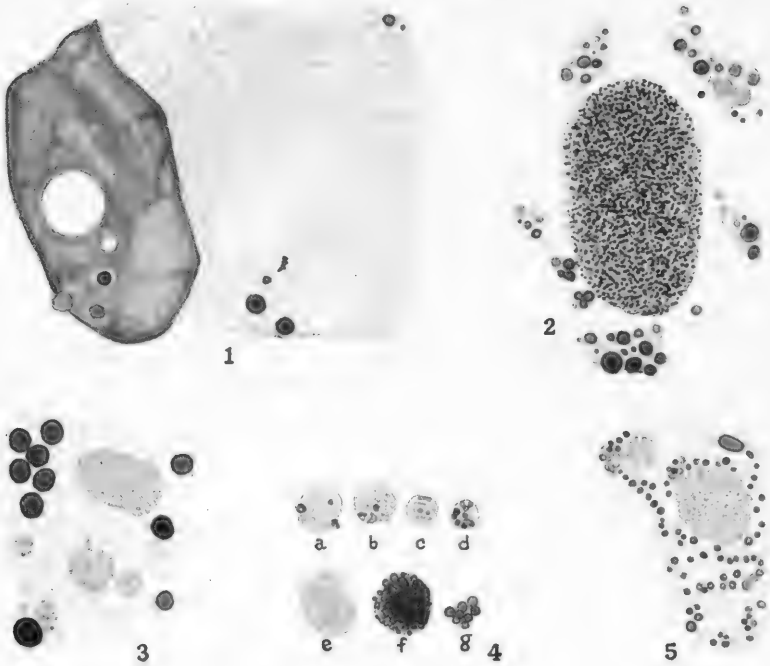
FIG. 10. Clumps of *Rickettsiæ* in a large intestinal epithelial cell. Close examination reveals the presence of rather larger atypical bodies (see page 263) embedded among the *Rickettsiæ*. From a nymph of Infective Series 607 taken 78 days after feeding as a larva and fixed in Regaud's fluid and colored by Giemsa's stain.

FIG. 11. Two clumps of *Rickettsiæ* within the lumen of the rectal sac. From

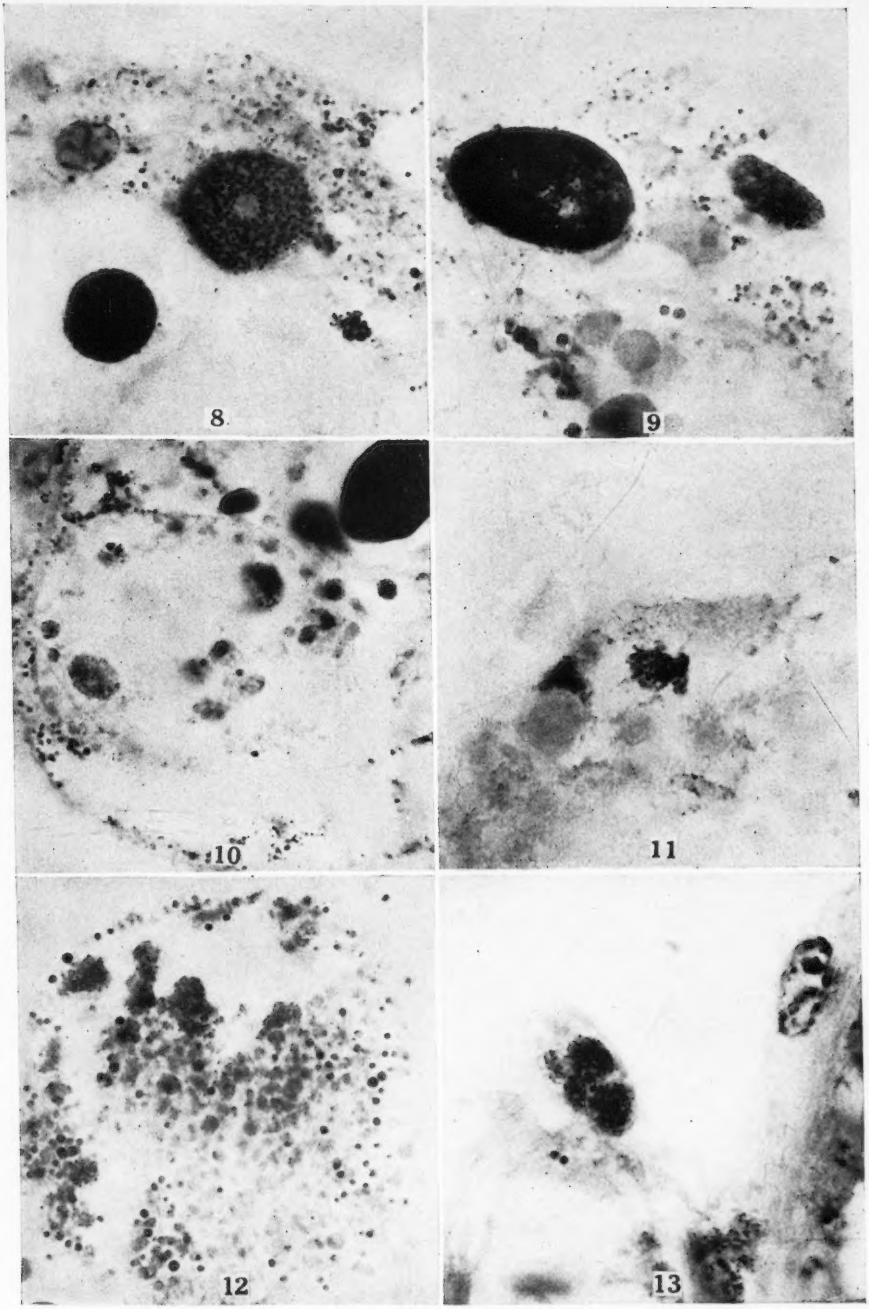
a nymph of Infective Series 607 taken 84 days after feeding as a larva, fixed in Carnoy's 6:3:1 fluid, and colored by Giemsa's stain.

FIG. 12. Large intestinal epithelial cell containing several clumps of *Rickettsiæ* embedded in a highly granular cytoplasm. From a nymph of Infective Series 607 which had fed on Goat 9651 and had been renumbered 637 (see Table IV). The viscera of the nymph were fixed in Regaud's fluid and colored by Giemsa's stain.

FIG. 13. Clumps of *Rickettsiæ* within an intestinal epithelial cell in an adult descended from a larva which 96 days previously had fed on an animal suffering from heartwater and which as a nymph fed upon Goat 9665, transmitting heartwater to it. Fixed in Regaud's fluid and colored by Giemsa's stain.



(Cowdry: Etiology of heartwater. II.)



(Cowdry: Etiology of heartwater. II.)